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Application of monoclonal antibodies to the investigation of the role of calcitonin gene-related peptide as a vasodilatory neurotransmitter

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Declaration

This dissertation is an account of my original work. However, a number of monoclonal antibodies were produced by others and made available to me as part of a research collaboration. These antibodies have been distinguished from those that I have produced, and their sources have been clearly stated. The characterization and application of these antibodies, reported in this dissertation, was entirely my own work.

I hereby declare that this dissertation entitled "Application of monoclonal antibodies to the investigation of the role of calcitonin gene-related peptide as a vasodilatory neurotransmitter" is not substantially the same as any that I have submitted for a degree, diploma or other qualification at any other University.

I further state that no part of my dissertation has already been or is being concurrently submitted for any such degree, diploma or other qualification.

Date..... 9 march 1994 ..... Signed.....

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Abbreviations

AUC	Area under the flux-time curve attributable to nerve stimulation
Bis	N'N'-Bis-methylene-acrylamide
BSA	Bovine serum albumin
B_{max}	Concentration of binding sites
CDR	Complementarity-determining region
CGRP	Calcitonin gene-related peptide
cpm	Counts per minute
95% C.I.	95% Confidence interval
DAB	3,3' Diaminobenzidine
DMEM	Dulbecco's modified Eagles medium
EC_{50}	Concentration which produces half-maximal effect
EDTA	Ethylene-diamine-tetraacetic acid
ELISA	Enzyme-linked immunoadsorbent assay
E_{max}	Maximum effect
2FD, 10FD, 20FD	Dulbecco's modified Eagles medium containing 2, 10, 20% foetal calf serum
FITC	Fluorescein isothiocyanate
Fmax	Maximum change in skin blood flow attributable to nerve stimulation
HAT	Hypoxanthine, aminopterin and thymidine
HαCGRP	Human α CGRP
$H\alpha CGRP_{8-37}$	C-terminal (8-37) fragment of HαCGRP
HβCGRP	Human β CGRP
HT	Hypoxanthine and thymidine
i.p.	Intraperitoneal
i.v.	Intravenous
KCl	Potassium chloride
Kd	Dissociation constant
MAb	Monoclonal antibody
MAP	Mean arterial pressure
NK_1	$Neurokinin_1$
PAbs	Polyclonal antibodies
PBS	Phosphate-buffered saline
PBSTx	0.1M PBS/0.3% Triton-X 100

PEG	Polyethylene glycol
PMSF	Phenylmethyl-sulphonyl-fluoride
RαCGRP	Rat α CGRP
RβCGRP	Rat β CGRP
RIA	Radioimmunoassay
rpm	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SFD	Serum-free Dulbecco's modified Eagles medium
SHR	Spontaneously hypertensive rat
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
TSH	Thyroid-stimulating hormone

Summary

Calcitonin gene-related peptide (CGRP) is produced by alternative mRNA processing of the calcitonin gene. It is a potent vasodilator and is localized in perivascular sensory neurons. The localization of CGRP-immunoreactivity in primary afferent neurons innervating many different tissues and the wide distribution of CGRP binding sites suggest that CGRP may be a physiologically important neurotransmitter. The aim of the project was to investigate whether vasodilatory responses to CGRP released from perivascular sensory nerves could be blocked with anti-CGRP or anti-CGRP receptor monoclonal antibodies (MAbs).

MAbs against CGRP were successfully produced and characterized for their ability to inhibit CGRP receptor binding (immunoblockade). Unsuccessful attempts were made to develop MAbs against the CGRP receptor by *in vivo* and *in vitro* immunization of animals with CGRP receptor-rich membranes and by an auto-anti-idiotypic approach.

Eleven MAbs against CGRP were screened for immunoblocking properties in an isolated porcine coronary artery assay or an electrically-stimulated isolated rat vas deferens assay. MAb C4.19 was identified as a MAb that effectively blocks the effects of exogenous rat CGRP. It was demonstrated that the pharmacological response to CGRP in the presence of MAb C4.19 could be predicted when the dissociation constant and concentration of binding sites of the antibody were known. Capsaicin was used to stimulate the release of endogenous CGRP from primary afferent neurons. Capsaicin-induced inhibition of electrically-stimulated contractions of the isolated rat vas deferens was significantly attenuated by MAb C4.19. The results of the *in vitro* immunoblockade studies suggest that CGRP has a major role as a neurotransmitter at the neuroeffector junction of the rat vas deferens.

The effect of MAb C4.19 on the hypotensive response to exogenous rat αCGRP (RαCGRP) was investigated in the pentobarbitone-anaesthetized rat. The role of CGRP in mediating antidromic vasodilatation was investigated by blockade of the increase in hind paw skin blood flow produced by saphenous nerve stimulation in the pentobarbitone-anaesthetized rat. Change in skin blood flow was measured by laser Doppler flowmetry. The dose-response relationship for the effect of i.v. RαCGRP was similarly shifted rightward by MAb C4.19 IgG (1 mg/rat i.v.) and Fab' fragment (2 mg/rat i.v.). The C-terminal fragment of human αCGRP ($H\alpha CGRP_{8-37}$; 100 nmol/kg i.v.) also blocked the hypotensive effect of RαCGRP significantly. MAb C4.19 Fab' fragment (2 mg/rat i.v.) and $H\alpha CGRP_{8-37}$ (100 nmol/kg i.v.) but not MAb C4.19 IgG (up to 3 mg/rat i.v.) blocked the increased skin blood flow response to antidromic stimulation of the saphenous nerve. Normal mouse Fab' fragment

(2 mg/rat i.v.) had no significant effect on antidromic skin vasodilatation. The mean percentage changes in skin blood flow parameters due to MAb C4.19 Fab' fragment were significantly different from those due to normal mouse Fab' fragment but not from those due to HαCGRP$_{8-37}$.

The results of this project show that immunoblockade with an anti-CGRP MAb may be used to demonstrate the physiological role of endogenous CGRP. However, Fab' fragments should be used for acute *in vivo* pharmacological studies to ensure effective distribution to the site of action. The results of immunoblockade agree with those obtained by receptor blockade with HαCGRP$_{8-37}$ and provide complementary evidence in support of the role of CGRP in mediating skin vasodilatation.

Correction (page 1, lines 24-25): "A CGRP ....." should read "A form of CGRP ....."

CHAPTER 1

General introduction

1.1. Calcitonin gene-related peptide

Calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide which was discovered after its structure and existence were predicted from molecular cloning studies of the rat calcitonin gene (Amara *et al.*, 1982; Rosenfeld *et al.*, 1983). Alternative processing of the primary mRNA transcript of the calcitonin gene leads to the production of calcitonin or CGRP. Human CGRP was first isolated from medullary thyroid carcinoma tissue (Morris *et al.*, 1984).

A second gene encoding a closely homologous 37-amino acid peptide was subsequently identified in rat and man (Amara *et al.*, 1985; Steenbergh *et al.*, 1985). The second peptide is termed βCGRP to distinguish it from the originally described CGRP, now referred to as αCGRP.

The calcitonin/αCGRP gene is transcribed as a larger precursor mRNA containing 6 exons which are subsequently spliced to form either calcitonin or αCGRP. The fully processed mRNAs have the first 3 exons in common but contain distinct 3' terminal exons. The transcripts from the βCGRP gene, however, are not processed to form multiple mRNAs. Thus the βCGRP gene does not code for a second calcitonin. The calcitonin/αCGRP and βCGRP genes are both located on chromosome 11.

1.1.1. Structure of CGRP

CGRP consists of 37 amino acids with an N-terminal 6-amino acid ring structure linked by a disulphide bridge and an amidated C-terminus. The two forms of CGRP differ in three amino acid positions in man but only one in the rat (Figure 1.1). A CGRP with striking homology to the rat and human CGRPs has also been isolated from the spinal cord of the pig (Kimura *et al.*, 1987).

CGRP shares limited structural similarities with calcitonin. The sequence homology between human αCGRP (HαCGRP) and human calcitonin is only 16%. The different forms of CGRP cross-react weakly with calcitonin receptors and are about 100- to 1000-fold less potent than human calcitonin in lowering plasma calcium levels and inhibiting osteoclastic activity (Zaidi *et al.*, 1988; Raue *et al.*, 1987). Salmon calcitonin at high concentrations can cross-react with CGRP receptors (Goltzman & Mitchell, 1985; Wimalawansa & El-Kholy, 1993).

Perhaps more significantly, there are major structural similarities between

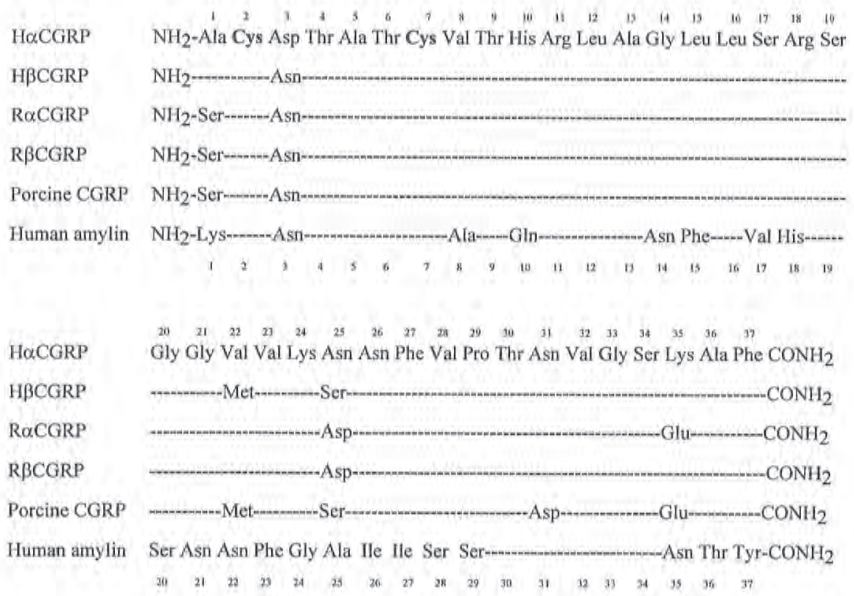

Figure 1.1: Structure of α and β forms of CGRP from man, rat and pig. The structure of human amylin is shown for comparison. Broken horizontal lines indicate regions of sequence identity with HαCGRP. Cysteine residues at positions 2 and 7 (in bold) take part in a disulphide bond.

CGRP and another 37-amino acid peptide called amylin which is secreted from pancreatic islets of Langerhans. Human amylin, also known as islet amyloid polypeptide, was originally isolated from the amyloid deposits of an insulinoma (Westermark *et al.,* 1986) and of Type II diabetic pancreases (Cooper *et al.,* 1987). It has 43% and 46% sequence homology with HαCGRP and human βCGRP (HβCGRP) respectively. Indeed, amylin shares many of the biological properties of CGRP and cross-reacts with CGRP receptors (Section 1.1.9.1.).

More recently, a novel 52 amino acid peptide showing slight sequence homology to CGRP has been isolated from human phaeochromocytoma (Kitamura *et al.,* 1993). This peptide has been named adrenomedullin. Nine amino acid residues in the C-terminal (15-52) end of adrenomedullin are also found in the α and β forms of human CGRP.

1.1.2. Distribution of CGRP

CGRP is widely distributed in the central and peripheral nervous systems (Tschopp *et al.*, 1984; Lee *et al.,* 1985; Wimalawansa, *et al.*, 1987). The presence of CGRP immunoreactivity has been detected primarily by immunocytochemistry and radioimmunoassay with anti-CGRP sera which do not distinguish between the two

known forms of CGRP. However, the expression of the α and β forms of CGRP has been revealed by hybridization histochemistry using specific RNA probes (Mulderry *et al.*, 1988; Noguchi *et al.*, 1990; Sternini & Anderson, 1992). Both forms of CGRP are localized primarily in neural tissues.

In the brain, CGRP is present in the nuclei of sensory and motor cranial nerves and in cell bodies in distinct regions including the hypothalamus, preoptic area, ventromedial thalamus, medial amygdala and hippocampus (Skofitsch & Jacobowitz, 1985a; Yamamoto & Tohyama, 1989). In the spinal cord, CGRP-immunoreactive fibres are distributed primarily in the dorsal horn which receives sensory input. Dorsal rhizotomy induces a marked loss of CGRP-immunoreactive fibres from the dorsal spinal cord. Thus the CGRP-containing fibres are central projections of afferent neurons originating from the dorsal root ganglion (Gibson *et al.*, 1984). The mRNAs of the α and β forms of CGRP are co-expressed in dorsal root ganglion cells (Noguchi *et al.*, 1990).

CGRP immunoreactivity is found within cells and sensory nerve fibres in diverse peripheral organs including the heart, lung, urogenital tract, tongue, pancreas, skin and gastrointestinal tract (Gibbins *et al.*, 1985; Wimalawansa *et al.*, 1987; Mulderry *et al.*, 1988). Throughout the body, it is localized in perivascular sensory nerve fibres (Rosenfeld *et al.*, 1983; Mulderry *et al.*; 1985, Uddman *et al.*, 1986). The density of fibres around arteries is generally higher than that around veins (Uddman *et al.*, 1986). Combined retrograde tracing and immunocytochemical studies have demonstrated that the CGRP-immunoreactive nerves in the periphery originate from dorsal root ganglia (Alm & Lundberg, 1988; Louis *et al.*, 1989; Sternini & Anderson, 1992). However, the origin of most of the CGRP-immunoreactive cerebrovascular nerve fibres appears to be the trigeminal ganglion. CGRP-immunoreactive fibres are found in the adventitia and the adventitial-medial border of blood vessels (Gulbenkian *et al.*, 1986; Edvinsson *et al.*, 1987; Shoji *et al.*, 1987).

CGRP-immunoreactive fibres are present in all regions of the heart, particularly in association with the coronary arteries, within the papillary muscles, and within the sinoatrial and atrioventricular nodes (Mulderry *et al.*, 1985). Tissue concentrations of immunoreactive CGRP are higher in the atria than the ventricles in rat and guinea pig hearts (Wharton *et al.*, 1986; Wimalawansa & MacIntyre, 1988). In human cardiopulmonary tissue, the highest levels of CGRP immunoreactivity are found in the left anterior descending coronary artery, followed in declining order by the bronchus, right atrium, pulmonary artery, lung and left ventricle (Franco-Cereceda, 1991). CGRP-immunoreactive nerve fibres are very sparse in the proximal region of human epicardial arteries but increases in number distally (Gulbenkian *et al.*, 1993).

CGRP immunoreactivity is also localized in non-nervous tissue. The peptide was originally reported to be absent in the rat thyroid gland (Rosenfeld *et al.*, 1983). However, CGRP immunoreactivity has been subsequently co-localized with calcitonin in thyroid C-cells (Sabate *et al.*, 1985; Lee *et al.*, 1985). Rat thyroid C-cells produce both calcitonin and CGRP mRNAs in a ratio of approximately 95:1. CGRP immunoreactivity is also localized in nerve fibres in the thyroid gland. In the lung, CGRP immunoreactivity is localized in capsaicin-sensitive nerve fibres and in endocrine cells (Cadieux *et al.*, 1986; Shimosegawa & Said, 1991). CGRP immunoreactivity has been localized in subpopulations of endothelial cells of term human umbilical vein and artery (Cai *et al.*, 1993).

CGRP is frequently co-localized with substance P in primary afferent neurons (Lee *et al.*, 1985; Lundberg *et al.*, 1985; Ju *et al.*, 1987; Quartu *et al.*, 1992). The co-existence of the two peptides in trigeminal ganglia, dorsal root ganglia and perivascular nerve fibres has been demonstrated at the ultrastructural (electron microscopical) level (Gulbenkian *et al.*, 1986). However, primary afferent neurons containing only one or other peptide also occur (Lee *et al.*, 1985; Quartu *et al.*, 1992), and CGRP may be co-localized with other peptides such as somatostatin in the sensory axons of human skin (Gibbins *et al.*, 1987).

In addition to its widespread distribution in sensory neurons, CGRP immunoreactivity is localized in motoneurons (Gibson *et al.*, 1984; Mora *et al.*, 1989). Only βCGRP mRNA appears to be expressed in enteric neurons, unlike sensory neurons which express both forms of CGRP mRNA (Mulderry *et al.*, 1988; Sternini & Anderson, 1992). In the autonomic nervous system, CGRP is present pre-ganglionically in sympathetic and parasympathetic nerve fibres (Kruger *et al.*, 1988).

1.1.3. Distribution of CGRP binding sites

Specific binding sites for CGRP are distributed in distinct regions of the brain (Henke *et al.*, 1985; Skofitsch & Jacobowitz, 1985b; Kruger *et al.*, 1988) and the spinal cord (Yashpal *et al.*, 1992). Diverse peripheral tissues contain specific binding sites including blood vessels, heart, liver, thyroid gland, pituitary gland, spleen, lungs, pancreas, penis, adrenal gland and bladder (Goltzman & Mitchell, 1984; Nakamuta *et al.*, 1986; Sigrist *et al.*, 1986, Wimalawansa *et al.*, 1987).

In the cardiovascular system of the rat, the highest concentrations of both CGRP and its binding sites are found in peripheral and mesenteric arteries (Wimalawansa & MacIntyre, 1988). Specific binding sites on blood vessels are distributed in the media and intima (Sigrist *et al.*, 1986). CGRP binding sites have been identified in cultured vascular smooth muscle cells and endothelial cells (Hirata

Table 1.1: Biological effects attributed to CGRP in the periphery

Effect	Reference
Vasodilatation	Brain *et al.*, 1985
Inotropic effect	Ishikawa *et al.*, 1988
Chronotropic effect	Marshall *et al.*, 1986
Potentiation of inflammatory oedema	Brain & Williams, 1985
Chronic inflammation	Louis *et al.*, 1990
Bronchoconstriction	Palmer *et al.*, 1987
Inhibition of leukotriene release from lung	Di Marzo *et al.*, 1986
Inhibition of insulin secretion	Ahren *et al.*, 1987
Inhibition of insulin-stimulated glycogen synthesis	Leighton & Cooper, 1988
Inhibition of gastrointestinal motility	Forster & Dockray, 1991
Inhibition of gastric acid secretion	Tache, 1992
Stimulation of gastric somatostatin release	Inui *et al.*, 1991
Stimulation of pancreatic amylase release	Seifert *et al.*, 1985
Inhibition of aldosterone secretion	Murakami *et al.*, 1989
Inhibition of urinary tract motility	Maggi *et al.*, 1992
Nicotinic receptor synthesis	New & Mudge, 1986
Nicotinic receptor desensitization	Mulle *et al.*, 1988
Inhibition of T-lymphocyte proliferation	Umeda *et al.*, 1989
T-lymphocyte chemotaxis	Foster *et al.*, 1992
Stimulation of endothelial cell proliferation	Haegerstrand *et al.*, 1990
Peripheral nerve regeneration	Dumoulin *et al.*, 1992
Regulation of calcium metabolism	Zaidi *et al.*, 1988
Intestinal ion transport	Cox *et al.*, 1989

et al., 1988). The density of specific CGRP binding sites may depend on vessel size. Specific binding sites are more abundant in small intramyocardial compared with large epicardial coronary arteries (Sun *et al.*, 1993).

1.1.4. Biological effects of CGRP

The widespread distribution of CGRP and its binding sites in the central and peripheral nervous systems suggests that it is a neuropeptide with multiple physiological roles. Indeed, CGRP has been shown to exhibit biological effects in virtually all organs. A detailed review of the diverse biological effects attributed to CGRP is beyond the scope of this introductory chapter. In accordance with the aim of

Correction (page 6, line 31):
"... can potent dilate ..." should read "... can potently dilate ..."

Table 1.2: Biological effects attributed to CGRP in the central nervous system

Effect	Reference
Stimulation of noradrenergic outflow	Fisher *et al.*, 1983
Hyperthermia	Dennis *et al.*, 1990
Decrease in food intake	Krahn *et al.*, 1984
Inhibition of gastrointestinal motility	Raybould *et al.*, 1988
Gastric mucosal protection	Tache, 1992
Inhibition of gastric acid secretion	Hughes *et al.*, 1984
Suppression of growth hormone release	Fahim *et al.*, 1990
Central nervous system differentiation	Denis-Donini, 1992
Antinociceptive effect	Pecile *et al.*, 1987
Lowering of nociceptive threshold	Oku *et al.*, 1987

the project (Section 1.3), the role of CGRP as a vasodilator will be reviewed below. However, the peripheral and central effects of CGRP have been summarized in Tables 1.1 and 1.2 respectively.

1.1.5. CGRP as a vasodilator

The first suggestion of the vasodilatory properties of CGRP came from observations by Fisher *et al.* (1983) that intravenous injection of RαCGRP produced dose-dependent hypotension and tachycardia in conscious rats. The vasodilatory activity of rat and human αCGRP was clearly established by Brain *et al.* (1985) in rabbit skin, human skin, isolated rat aorta and the hamster cheek pouch microvasculature. Moreover, HαCGRP appeared to be more potent at lowering blood pressure when infused i.v. to normal volunteers than any other vasodilator (Struthers *et al.*, 1986). Lappe *et al.* (1987) demonstrated that RαCGRP increased cardiac output and reduced total peripheral resistance simultaneously in conscious rats. Therefore, the hypotensive effect of CGRP is mediated through peripheral vasodilatation rather than through reductions in cardiac output. Since these original observations, the potent vasodilatory activity of CGRP has been widely confirmed in man and in all animal species studied, whether in isolated blood vessels (strips or rings), isolated perfused vascular beds or *in vivo*. Examples are shown in Table 1.3. Although regional and species variations exist, it is noteworthy that CGRP can potent dilate both large vessels and arterioles which are more relevant to the regulation of peripheral vascular resistance. Compared with other vasodilators, and substance P in particular, CGRP produces a long-lasting vasodilatation. Following brachial artery infusion in man, its

Table 1.3: Examples of the vasodilatory response to CGRP observed in various vascular beds and species

Blood vessel/vascular bed	Species	Reference
Renal arterioles	rabbit	Edwards & Trizna, 1990
Skin	human	Brain *et al.*, 1986a
Cerebral artery	human	Edvinsson *et al.*, 1987
Nasal mucosa	pig	Stjarne *et al.*, 1991
Coronary arteries	pig	Franco-Cereceda *et al.*, 1987a
Pulmonary artery	human	McCormack *et al.*, 1989
Mesenteric arterial bed	rat	Kawasaki *et al.*, 1988
Opthalmic artery	pig	Bakken *et al.*, 1992
Uterine artery	human	Nelson *et al.*, 1993a
Gastric submucosa	rat	Chen *et al.*, 1992
Synovial vessels	rat	Cambridge & Brain, 1992
Skeletal muscle arterioles	rabbit	Ohlen *et al.*, 1987
Tracheal artery	dog	Salonen *et al.*, 1988

half-life of biological effect (increased forearm blood flow) was approximately 18 minutes in contrast to a half-life of biological effect of approximately 15 seconds estimated for substance P (McEwan *et al.*, 1988).

The β form of CGRP is also a potent vasodilator (Brain *et al.*, 1986b). Both similar and differential vasodilatory effects have been reported for the two forms of CGRP. Approximately equipotent vasodilatation have been reported in animals and in man *in vitro* (Franco-Cereceda *et al.*, 1987a; Franco-Cereceda, 1991) and *in vivo* (Zaidi *et al.*, 1990; Hughes & Brain, 1991; Williams *et al.*, 1988). On the other hand, studies in conscious rats have suggested that HαCGRP and HβCGRP may differ in some of their regional haemodynamic effects and that these differences may be dose-dependent (Gardiner *et al.*, 1989). HβCGRP has been reported to be a more potent coronary vasodilator than HαCGRP in the rat (Holman *et al.*, 1986) but this does not appear to be the case in man (Franco-Cereceda, 1991). More potent *in vivo* vasodilatory effects have been reported for HαCGRP compared with HβCGRP in man (Beglinger *et al.*, 1991). RαCGRP has been reported to be 3 times more potent than RβCGRP as a dilator of the isolated perfused bed of the rat left gastric artery (Holzer *et al.*, 1993).

The discrepancies in results reported by various workers may reflect species and regional differences in sensitivity to αCGRP and βCGRP. Differences in the metabolic degradation of the two forms of CGRP have been suggested as an

explanation of the their different potencies *in vivo* but the clearance of HαCGRP and HβCGRP in man have been reported to be similar (Beglinger *et al.*, 1991). On balance, the vasodilatory properties of αCGRP and βCGRP are characterized by their similarity rather than their differences; in general, the differences reported are not large enough (e.g., 10-fold) to be physiologically important.

CGRP may be a potent but selective vasodilator in different vascular beds. The most pronounced increases in blood flow have been detected in the skin and gastric circulations following i.v. administration of CGRP to conscious rats (Ando *et al.*, 1990). Comparison of haemodynamic measurements in different vascular beds indicate that CGRP is a selective dilator in the carotid vascular bed in the rat (Gardiner *et al.*, 1989) and in man (MacDonald *et al.*, 1989; Mulholland *et al.*, 1991). CGRP appears to redistribute blood flow to the skin and carotid artery at the expense of mesenteric blood flow in man (Jager *et al.*, 1990; Mulholland *et al.*, 1991). Differential vasodilatory responses to CGRP have been reported within the coronary arterial bed, depending on the diameter of conduit and resistance vessels studied (Foulkes *et al.*, 1991; Ludman *et al.*, 1991; Sekiguchi *et al.*, 1994). In the kidney, CGRP dilates renal afferent but not efferent arterioles (Edwards & Trizna, 1990).

There is no doubt that CGRP is a potent arterial dilator. However, the evidence for CGRP-induced dilatation of veins is less clear-cut. CGRP relaxes isolated rat femoral (Edvinsson *et al.*, 1989b), human pial (Hardebo *et al.*, 1987) and human pulmonary veins (McCormack *et al.*, 1989) but not isolated human saphenous veins (Marshall *et al.*, 1988). Infusion of CGRP into the brachial artery in man leads to a marked and prolonged increase in forearm blood flow but CGRP does not dilate pre-constricted superficial veins of the hand (McEwan *et al.*, 1988). CGRP is a potent dilator of the rat isolated perfused mesenteric vasculature on the arterial side but is a weak dilator on the venous side (Claing *et al.*, 1992). In the anaesthetized dog, CGRP increases blood flow more potently in the perfused hepatic arterial than the portal vascular bed (Withrington *et al.*, 1992). Thus there is good evidence that CGRP is less potent as a venous than an arterial dilator. The role of CGRP as a physiological venous dilator is, however, supported by the finding in conscious rats that it decreased mean circulatory filling pressure (an index of body venous tone); this was particularly evident when venous tone was elevated (Abdelrahman & Pang, 1992).

1.1.5.1. Mechanisms of vascular relaxation

Several mechanisms have been proposed for the mechanism of CGRP-induced vasodilatation. The vasodilatory effect of CGRP is not mediated by adrenergic, cholinergic or histaminergic mechanisms (Hanko *et al.*, 1985; Franco-Cereceda *et al.*, 1987a). However, both endothelium-dependent and -independent mechanisms have

been reported. CGRP has also been reported to act as a vasodilator by activation of adenosine 5'-triphosphate (ATP)-sensitive potassium (K_{ATP}) channels (Nelson *et al.*, 1990).

Examples of blood vessels in which vasodilatation is endothelium-independent include the isolated pial artery of the rabbit, cat and man (Hanko *et al.*, 1985), canine cerebral artery (Ikegaki *et al.*, 1989), rabbit hepatic artery (Brizzolora & Burnstock, 1991), human uterine artery (Nelson *et al.*, 1993a) and human coronary artery (Franco-Cereceda, 1991). The endothelium-independent relaxation of isolated porcine coronary artery rings is associated with adenylate cyclase activation and a decrease in intracellular free calcium concentration (Shoji *et al.*, 1987; Kageyama *et al.*, 1993). In contrast, relaxation of the isolated rat aorta is dependent on the presence of an intact endothelium (Brain *et al.*, 1985; Grace *et al.*, 1987). CGRP has been shown to mediate endothelium-dependent relaxation of rat aortic rings by a novel signal transduction mechanism involving activation of both adenylate cyclase and guanylate cyclase (Gray & Marshall, 1992; Wang *et al.*, 1991). The endothelium-derived relaxing factor released by CGRP is probably nitric oxide because the relaxation of aortic rings is blocked by inhibitors of nitric oxide synthase.

CGRP relaxes pre-contracted segments of some human arteries, including radial, gastric and cerebral arteries, in an endothelium-dependent manner (Thom *et al.*, 1987). Whether CGRP-induced vasodilatation is endothelium-dependent may depend on the size of the vessel (Hughes *et al.*, 1988). The relaxation of human resistance arteries by CGRP does not depend on the integrity of the endothelium.

Endothelium-dependent and -independent mechanisms may operate concurrently within a vascular bed. Prieto *et al.* (1991) reported that the relaxation of isolated rat proximal epicardial but not distal intramyocardial coronary artery rings is endothelium-dependent. Both nitric oxide-dependent and -independent mechanisms have been implicated in the hyperaemic action of CGRP in the rat gastric circulation (Holzer *et al.*, 1993).

The hypotensive effect of RαCGRP in conscious rats is attenuated by nitric oxide synthase inhibitors (Abdelrahman *et al.*, 1992). Differential contribution of nitric oxide to the regional vasodilatory effects of CGRP has been demonstrated; CGRP-mediated vasodilatation of the hindquarters vascular bed has a substantial nitric oxide-dependent component (Gardiner *et al.*, 1991c).

In addition to mediating endothelium-dependent CGRP vasorelaxation, there is evidence that nitric oxide may modulate the release of CGRP or act as a vasodilatory neurotransmitter in its own right. The nitric oxide synthase inhibitor L-nitro-arginine methyl ester does not affect the vasodilatory effect of HαCGRP but significantly inhibits capsaicin-induced vasodilatation in rabbit skin (Brain *et al.*,

1993; Hughes & Brain, 1994). Nitric oxide, rather than CGRP, may mediate relaxation induced by electrical stimulation of bovine basilar artery (Ayajiki *et al.*, 1993).

There is evidence that prostaglandins could mediate the endothelium-dependent vasodilatory effects of CGRP. The peptide is capable of releasing prostacyclin from human umbilical vein endothelial cells (Crossman *et al.*, 1987). The relaxation of rat aortic rings by CGRP has been reported to be partially inhibited by the cyclooxygenase inhibitor indomethacin (Brain *et al.*, 1985). However, further studies failed to demonstrate inhibition of CGRP-induced relaxation of pre-constricted rat aortic rings or cyclic nucleotide responses with ibuprofen or indomethacin. (Gray & Marshall, 1992; Fiscus *et al.*, 1991). The reasons for these contradictory results are unclear. Prostaglandins may play a role in mediating the renal haemodynamic effects of HβCGRP. The increased renal blood flow response to intrarenal infusion of HβCGRP in anaesthetized dogs is markedly attenuated by treatment with indomethacin (Villarreal *et al.*, 1988). The use of indomethacin has also implicated the involvement of prostaglandins in mediating CGRP-induced endothelium-dependent relaxation of rat proximal epicardial coronary artery rings (Prieto *et al.*, 1991).

There is conflicting evidence on the role of K_{ATP} channels in mediating the vasodilatory effects of CGRP. Partial blockade of CGRP vasodilatation and arterial smooth muscle hyperpolarization by the K_{ATP} channel antagonist glibenclamide was originally observed in the isolated rabbit mesenteric artery (Nelson *et al.*, 1990). Some evidence has accumulated in support of the role of K_{ATP} channels including glibenclamide blockade of CGRP-induced vasodilatation of the isolated human uterine artery (Nelson *et al.*, 1993b), the rat basilar artery *in vivo* (Kitazono *et al.*, 1993) and the feline pulmonary vascular bed (Hood *et al.*, 1991). CGRP-induced hypotension is attenuated by glibenclamide in the rabbit (Andersson, 1992) but not in the rat (Abdelrahman *et al.*, 1992). Glibenclamide does not significantly affect the vasodilatory effect of CGRP in the isolated perfused rat lung (Tjen-A-Looi *et al.*, 1992) and the isolated porcine coronary artery (Kageyama *et al.*, 1993), rabbit ophthalmic artery (Zschauer *et al.*, 1992), rat coronary artery (Prieto *et al.*, 1991) and human mammary artery (Boyle & Brown, 1991).

The different mechanisms reported for the vasodilatory action of CGRP is likely to reflect species and regional variation, receptor heterogeneity, the use of different experimental conditions (e.g., *in vitro* or *in vivo*), or a combination of these factors.

1.1.6. Effects of CGRP on the heart

Positive chronotropic and inotropic effects of CGRP are observed in the isolated rat and guinea pig right atrium (Marshall *et al.*, 1986; Franco-Cereceda & Lundberg, 1985). However, some species differences may exist; CGRP has positive chronotropic effects on rat and guinea pig, but not rabbit, isolated perfused hearts (Holman *et al.*, 1986; Franco-Cereceda & Lundberg, 1985). Rat αCGRP and βCGRP are equipotent chronotropic and inotropic agents in isolated rat atria (Mulderry *et al.*, 1988). The effects of CGRP on isolated atria are dependent on adenylate cyclase activation (Ishikawa *et al.*, 1988), and may be observed in the presence of α- and β-adrenergic, histaminergic and muscarinic receptor antagonists (Franco-Cereceda & Lundberg, 1985; Saito *et al.*, 1987).

It is difficult to unequivocally establish a direct chronotropic effect of CGRP *in vivo* because a tachycardia inevitably accompanies hypotension through the activation of baroreflexes. Gardiner *et al.* (1989) attempted but failed to demonstrate chronotropic effects independently of any change in mean blood pressure in conscious rats. However, tachycardia in the absence of a fall in blood pressure has been reported in healthy volunteers (MacDonald *et al.*, 1989). The effect of β-blockade on the positive chronotropic effect of i.v. CGRP seems to vary between species. The tachycardia accompanying CGRP-induced hypotension has been reported to be abolished by β-adrenoceptor antagonists in conscious dogs (Wang *et al.*, 1989), attenuated in conscious rats (Lappe *et al.*, 1987; Siren & Fuerstein, 1988) and unchanged in healthy man (Gennari *et al.*, 1985).

Removal of baroreflexes by sinoaortic denervation results in enhanced vasodilatory responses but attenuated chronotropic responses in conscious rats (Siren & Fuerstein, 1988). In the anaesthetized rabbit, the chronotropic response to CGRP is markedly attenuated by sinoaortic plus vagal deafferentation and by restoration of arterial blood pressure with phenylephrine (Okamoto *et al.*, 1992). CGRP-induced tachycardia may be markedly attenuated by the ganglion blocker hexamethonium (Abdelrahman & Pang, 1992). In contrast, Haas & Skofitsch (1985) observed CGRP-induced tachycardia in the pithed rat, an animal model devoid of baroreflex mechanisms. The contradictory data have probably arisen from species variation and variable experimental conditions. It is likely that the chronotropic response to CGRP *in vivo* is due in part to baroreceptor-mediated reflexes and in part to a direct action of CGRP on the heart.

Correction (page 12, lines 11-12):

"...which prevents the interaction between receptor and G-protein ..." should read "...a non-hydrolyzable guanine nucleotide which prevents the formation of high affinity ligand:receptor:G protein ternary complexes ..."

1.1.7. Functional aspects of CGRP receptors

1.1.7.1. Receptor-effector coupling

Stimulation of CGRP receptors leads to activation of adenylate cyclase in various tissues and cells. For example, in the cardiovascular system, CGRP receptors are functionally coupled to adenylate cyclase in the atrium (Sigrist *et al.*, 1986), coronary arteries (Shoji *et al.*, 1987), intracerebral arterioles (Edwards *et al.*, 1991), cultured vascular smooth muscle cells and endothelial cells (Hirata *et al.*, 1988; Crossman *et al.*, 1987). However, CGRP receptors in the rat spinal cord, brainstem and whole brain are not coupled to adenylate cyclase (Goltzman & Mitchell, 1985; Stangl *et al.*, 1993; Semark *et al.*, 1992).

Guanosine 5'-o-(3-thiotriphosphate) (GTP-γ-S), which prevents the interaction between receptor and G-protein, has been reported to decrease the affinity of CGRP binding to rat liver, whole brain, cerebellum, spleen and neonatal cardiac myocyte membrane preparations (Yamaguchi *et al.*, 1988; Semark *et al.*, 1992; Chatterjee & Fisher, 1991; Stangl *et al.*, 1993; Chatterjee *et al.*, 1991). Thus the CGRP receptor in these preparations is probably linked to a G-protein. Chatterjee *et al.* (1993) found that a significant portion of soluble CGRP receptors (from rat cerebellum) could be immunoprecipitated with an antiserum against the amino-terminal region of $G_{s\alpha}$. This is the first evidence that CGRP receptors could be coupled to G_s. Photoaffinity labelling studies indicate that CGRP receptors are likely to be 60 to 70 kDa, glycosylated and with internal disulphide bonds (reviewed by Poyner, 1992)

1.1.7.2. Receptor antagonists

No non-peptide antagonist of CGRP has yet been reported. The most widely used CGRP antagonist is the C-terminal 8-37 fragment of HαCGRP (HαCGRP$_{8-37}$) which was first shown to antagonize CGRP activation of adenylate cyclase in rat liver membranes (Chiba *et al.*, 1989). Shorter C-terminal fragments also behave as CGRP antagonists but the residues in positions 9 to 12 are important for the maintenance of potent antagonistic properties (Mimeault *et al.*, 1991, 1992). The C-terminal 19-37 and 23-37 fragments retain weak but significant antagonistic activity in the guinea pig isolated left atrium assay (Rovero *et al.*, 1992). Antagonistic activity of the tyrosinated C-terminal 28-37 fragment of CGRP has also been reported in the opossum internal anal sphincter (Chakder & Rattan, 1990). However, unlike HαCGRP$_{8-37}$, the 28-37 fragment does not block HαCGRP-induced relaxation of pre-contracted isolated guinea-pig basilar artery segments (Jansen, 1992) nor the carotid vasodilatory effects of HαCGRP in conscious rats (Gardiner *et al.*, 1991a).

Several findings suggest that HαCGRP$_{8-37}$ is not an ideal CGRP antagonist,

apart from its peptide nature. Wide variation in its antagonistic potency has been reported in functional CGRP assays using the same tissue (reviewed by Poyner, 1992). In small diameter rings of the isolated porcine coronary artery that do not show tachyphylaxis to the effects of CGRP, the antagonistic potency of HαCGRP$_{8-37}$ was decreased by repeated exposure of tissues to the fragment. The gradient of the slope of the Schildt plot in this preparation was significantly less than 1 (Foulkes *et al.*, 1991). Non-competitive antagonist behaviour has been reported in the isolated rat aorta (Gray *et al.*, 1991) and intracerebral arterioles (Edwards *et al.*, 1991). Hα CGRP$_{8-37}$ is a weak calcitonin receptor agonist (Chiba *et al.*, 1989). Vasodilatory effects occasionally associated with high doses of HαCGRP$_{8-37}$ suggest that it may be a partial agonist of CGRP receptors (Hughes & Brain, 1991). In a photoaffinity labelling study of the CGRP receptor solubilized from human cerebellum, ^{125}I-HαCGRP$_{8-37}$ was crosslinked to a 95 kDa protein in addition to binding proteins labelled by the intact peptide (Stangl *et al.*, 1991). Thus HαCGRP$_{8-37}$ has the potential to cross-react with proteins not involved in CGRP binding.

Some of the discrepancies between early and later studies may be attributable to the quality of HαCGRP$_{8-37}$ used; improvements in synthetic techniques appear to have yielded a more pharmacologically active compound (Escott & Brain, 1993). It is unclear, however, to what extent different sources of supply and variable quality of peptide could explain the inconsistent findings on HαCGRP$_{8-37}$ as a competitive CGRP receptor antagonist.

1.1.7.3. Receptor subtypes

Evidence for the existence of CGRP receptor subtypes has been derived primarily from the differential potencies of the agonist [acetamidomethylcysteine$_{2,7}$]-HαCGRP and the C-terminal fragment antagonists HαCGRP$_{8-37}$ to HαCGRP$_{12-37}$ in a variety of *in vitro* and *in vivo* assays (Dennis *et al.*, 1989). CGRP receptors have been divided into two subclasses: CGRP$_1$ receptors (typically in guinea pig atria) which are sensitive to blockade with HαCGRP$_{8-37}$ and CGRP$_2$ receptors (typically in rat vas deferens) which are markedly less sensitive to blockade with HαCGRP$_{8-37}$ (Dennis *et al.*, 1990). The linear analogue [acetamidomethylcysteine$_{2,7}$]-HαCGRP, on the other hand, is a selective agonist at the CGRP$_2$ receptor (Dennis *et al.*, 1989). An additional third class of binding site has also been reported in the nucleus acumbens which can recognize HαCGRP, HαCGRP$_{8-37}$, [acetamidomethylcysteine$_{2,7}$]-HαCGRP and salmon calcitonin with high affinities (Sexton *et al.*, 1988; Dennis *et al.*, 1991).

Evidence for CGRP receptor heterogeneity based on differential potency in various tissues from different species must be viewed with caution. However, there is

evidence that receptor heterogeneity may exist within an individual tissue. Foulkes *et al.* (1991) provided evidence for receptor heterogeneity along the length of the porcine coronary arteries by demonstrating greater vasodilatory potency of CGRP, lack of development of tolerance to CGRP and greater antagonistic potency of Hα $CGRP_{8-37}$ in small diameter rings compared with large diameter rings. Gardiner *et al.* (1991a) found that HαCGRP-induced vasodilatation in the hindquarters of conscious rats was more sensitive to Hα$CGRP_{8-37}$ antagonism compared with the carotid vascular beds; such differences may be due to receptor heterogeneity.

The differential ability of CGRP to activate adenylate cyclase in membranes from different tissues (Semark *et al.*, 1992; Stangl *et al.*, 1993) also point to the existence of multiple receptors with different second messenger systems. Two populations of binding sites have been demonstrated in some but not all equilibrium radioligand binding studies (reviewed by Poyner, 1992); discrepancies have probably arisen from the wide range of experimental conditions used in different studies.

The binding site identified in human and bovine endothelial cells has an affinity at least 100-fold lower than CGRP receptors in other tissues (Crossman *et al.*, 1987; Hirata *et al.*, 1988; McEwan *et al.*, 1989). It has been suggested to be a distinct receptor subtype (McEwan *et al.*, 1989). However, human calcitonin cross-reacts with this receptor at relatively low concentrations (2 to 3 times molar excess; Hirata *et al.*, 1988) and doubts have been expressed on whether a CGRP "receptor" has been identified (Poyner, 1992).

There are inconsistent reports on the differential ability of Hα$CGRP_{8-37}$ to block responses to HαCGRP and HβCGRP. In rabbit skin, Hα$CGRP_{8-37}$ was a more potent antagonist of the vasodilatation induced by HαCGRP than HβCGRP (Hughes & Brain, 1991). Hα$CGRP_{8-37}$ has been found to block HαCGRP but not HβCGRP-induced dilatation of guinea-pig and human cerebral arteries (Jansen, 1992; Jansen *et al.*, 1992). However, the differential sensitivity of HαCGRP and HβCGRP-mediated responses to Hα$CGRP_{8-37}$ antagonism could not be confirmed in the isolated rabbit hepatic and dog basilar arteries (Butler *et al.*, 1993). It is not unclear at present whether HαCGRP and HβCGRP are selective agonists of different CGRP receptor subtypes.

Nuki *et al.* (1994) have recently provided functional evidence for the existence of presynaptic CGRP receptors on perivascular CGRP-containing nerves. In the isolated perfused rat mesenteric arterial bed with active tone produced by methoxamine plus guanethidine, perfusion of CGRP inhibited the vasodilatory response to periarterial nerve stimulation but had no effect on the vasodilatory response to bolus infusion of CGRP. The inhibitory effect of CGRP was antagonized by Hα$CGRP_{8-37}$. These findings suggest that CGRP-containing nerves may be

Correction (page 15, line 4): "Nuke *et al.*," should read "Nuki *et al.*"

endowed with presynaptic receptors that regulate CGRP release from the nerves via a negative feedback mechanism. Unlike postsynaptic $CGRP_1$ receptor-mediated responses, elevation of cyclic AMP does not appear to be responsible for the inhibitory effect of CGRP on neurogenic vasodilatation, and Nuke *et al.* have proposed that the presynaptic CGRP receptor may be a subtype of the $CGRP_1$ receptor.

1.1.8. CGRP as a neurotransmitter

1.1.8.1. Criteria for a neurotransmitter

A number of criteria must be fulfilled before CGRP could be established as an endogenous vasodilatory neurotransmitter. The classical criteria for the identification of a neurotransmitter include: (1) presence of the putative transmitter in terminal axons of appropriate nerves, (2) presence of systems for synthesis and storage, (3) release of putative transmitter by nerve stimulation, (4) mimicry of the response to nerve stimulation by the putative transmitter through receptor occupation, (5) pharmacological modification of the responses to the putative transmitter should have corresponding effects on the responses to nerve stimulation, and (6) presence of mechanisms for terminating the action of the released transmitter (Burnstock, 1986; Bowman & Rand, 1980).

1.1.8.2. Capsaicin

A considerable body of evidence concerning the storage and release of CGRP from nerves and the mimicry of the effects of nerve stimulation by CGRP has been derived from the use of capsaicin as a pharmacological tool. Capsaicin (8-methyl-N-vanillyl-6-noneamide) is the major pungent ingredient of hot peppers of the plant genus *Capsicum*. The neurophysiological and neurochemical effects of capsaicin are highly selective for unmyelinated C-fibres and thinly myelinated Aδ-fibres. Treatment of neonatal, but not adult, rats with the compound leads to degeneration and loss of the majority of primary afferent fibres. Capsaicin is more specific in stimulating C-type fibres than electrical nerve stimulation (Buck & Burks, 1986). The specific action of capsaicin on sensory neurons is mediated by the opening of a membrane cation channel that allows the movement of Ca^{2+} and Na^{+} into cells. A characteristic feature of the specific action of capsaicin on primary afferent neurons is its susceptibility to desensitization.

1.1.8.2.1. Release of CGRP from capsaicin-sensitive nerves

Capsaicin has been widely used as a pharmacological tool to investigate the "efferent" function of primary afferent neurons (reviewed by Maggi & Meli, 1988). Stimulation of capsaicin-sensitive sensory neurons leads to release of neuropeptides stored in nerve terminals which may, in turn, mediate various efferent functions. The usefulness of capsaicin as a tool for investigating the role of endogenous CGRP is based on evidence that capsaicin releases CGRP from sensory neurons *in vitro* and *in vivo*.

In vitro studies have demonstrated that CGRP is released from perivascular capsaicin-sensitive nerves in the rat and man (Fujimori *et al.*, 1990; Kawasaki *et al.*, 1990a; Del Bianco *et al.*, 1991; Franco-Cereceda, 1991). The release of CGRP from nerve terminals is calcium-dependent (Fujimori *et al.*, 1989; Franco-Cereceda, 1991). A 15-fold rise in plasma CGRP levels has been demonstrated after an intraperitoneal injection of 10 mg/kg capsaicin in rats (Emson & Zaidi, 1989). Neonatal treatment with capsaicin leads to a widespread but variable decrease of CGRP content in central and peripheral tissues of mature rats (Mulderry *et al.*, 1985; Wharton *et al.*, 1986; Diez Guerra *et al.*, 1988; Kashiba *et al.*, 1990; Wimalawansa, 1993). Plasma CGRP levels are significantly lower throughout the lifespan of neonatally treated rats (Wimalawansa, 1993). However, denervation hypersensitivity to the vasodilatory effects of CGRP have not been observed in rats treated neonatally with capsaicin (Bachelard *et al.*, 1992; Ralevic *et al.*, 1992; McEwan *et al.*, 1993).

It is noteworthy that, in addition to capsaicin, a wide range of drugs and endogenous substances could increase (e.g., prostaglandins and bradykinin) or decrease (e.g., adenosine and opioids) the excitability of primary afferent neurons with corresponding effects on CGRP release (reviewed by Maggi & Meli, 1988). Thus the effector functions of endogenous CGRP may be regulated prejunctionally. CGRP itself may have a role in modulating neurotransmission due to other transmitters. For example, CGRP mimics the effect of capsaicin in prejunctionally modulating purinergic transmission in the guinea-pig vas deferens (Ellis & Burnstock, 1989).

1.1.8.2.2. Non-specific effects of capsaicin

In addition to its selective action on primary afferent neurons, capsaicin also has cell non-selective effects that are usually manifested as a transient depression of excitability (Holzer, 1991). The concentrations needed to elicit these cell non-selective effects are typically orders of magnitude higher than those sufficient to stimulate sensory neurons. Cell non-selective effects of capsaicin include inhibition of

cardiac muscle excitability, inhibition of visceral smooth muscle activity and contraction of vascular smooth muscle. These non-selective effects tend to be sustained, not subject to desensitization, and are readily reproducible on reapplication of capsaicin. Application of capsazepine, the recently discovered competitive capsaicin antagonist, should allow better discrimination of the specific and non-specific actions of capsaicin (Bevan *et al.*, 1992).

The use of capsaicin as a tool for releasing endogenous CGRP is limited by the non-selective release of a number of neuropeptides found in primary afferent neurons which include substance P, neurokinin A, vasoactive intestinal polypeptide, somatostatin, cholecystokinin, corticotropin releasing factor, arginine vasopressin, bombesin, and galanin (Buck & Burks, 1986; Holzer, 1988; Maggi & Meli, 1988). Consideration must therefore be given to the extent to which CGRP, rather than other neuropeptides, mimics responses attributable to nerve stimulation by capsaicin. The problem of capsaicin-induced multiple neuropeptide release also highlights the need for specific blockers of the action of individual neuropeptides.

1.1.8.3. Metabolism of CGRP

The metabolism of CGRP is considered here because a major criterion for a neurotransmitter is that mechanisms for the inactivation of the putative neurotransmitter should exist. Substance P may have a role in regulating the vasodilatory activity of CGRP by stimulation or release of mast cell proteases which degrade CGRP (Brain & Williams, 1988). CGRP is effectively cleaved *in vitro* by human mast cell lysates and purified mast cell tryptase with loss of vasodilatory activity (Walls *et al.*, 1992). The degradation of CGRP by mast cell lysates and tryptase was similarly inhibited by a range of enzyme inhibitors. Thus it has been suggested that tryptase-catalysed CGRP metabolism may be an important mechanism by which the activity of CGRP is regulated *in vivo*.

CGRP has been reported to be metabolized by a substance P degrading endopeptidase in cerebrospinal fluid (Le Greves *et al.*, 1989). However, this enzyme is unlikely to be neutral endopeptidase (E.C.3.4.24.11) which cleaves HαCGRP at different sites. Neutral endopeptidase has been shown to cleave HαCGRP approximately 88 fold less rapidly than substance P (Katayama *et al.*, 1991).

The cleavage of CGRP to fragments with antagonistic activity may be another way of regulating its activity. One CGRP fragment with antagonistic activity, the C-terminal 19-37 fragment (Rovero *et al.*, 1992), has been identified as a major product of CGRP metabolizing peptidases in the rat central nervous system (Sakurada *et al.*, 1991).

1.1.9. Structurally-related peptides

1.1.9.1. Amylin

Considerable interest has been focused on amylin since its discovery because of its potential pathophysiological role in diabetes mellitus (Johnson *et al.*, 1989). Amylin may counteract the effects of insulin by decreasing second phase insulin secretion, increasing hepatic glucose output, and inhibiting insulin effects on skeletal muscle (Edwards & Morley, 1992). There is clear evidence that amylin cross-reacts with CGRP receptors in rat liver and skeletal muscle membranes (Morishita *et al.*, 1990; Chantry *et al.*, 1991). However, discrepancies in the potency of amylin and CGRP in mediating a range of common effects point to the existence of separate receptors for the two structurally-related peptides (Young *et al.*, 1993). Kreutter *et al.* (1993) showed that, unlike CGRP, the inhibition of insulin-stimulated glucose transport by amylin in isolated soleus muscle was independent of adenylate cyclase stimulation - this effect was not antagonized by HαCGRP$_{8-37}$. Compared with CGRP, amylin has higher potency for adenylate cyclase stimulation in the CHO-K1 cell line (D'Santos *et al.*, 1992) and higher affinity for distinct binding sites in the nucleus accumbens region of rat brain (Beaumont *et al.*, 1993). Comparison of the ability of HαCGRP$_{8-37}$ to antagonize the effects of RαCGRP, RβCGRP and amylin in the isolated guinea-pig left atrium, guinea-pig bladder and rat vas deferens also suggested that amylin is a selective agonist of different CGRP receptor subtypes or its own unique receptor (Giuliani *et al.*, 1992).

Amylin has vasodilatory effects that are significantly weaker than those of CGRP. In rabbit skin, human amylin is about a 100-fold less active as a vasodilator than HαCGRP (Brain *et al.*, 1990). Rat amylin is about 44-fold less potent than RαCGRP as a hypotensive agent in anaesthetized rats (Young *et al.*, 1993). The haemodynamic effects of rat amylin in conscious rats are similar to those of RαCGRP but they are observed at 100-fold higher doses (Gardiner *et al.*, 1991b). It is likely that these effects are mediated via interaction with CGRP receptors since they could be antagonized by HαCGRP$_{8-37}$. However, it is possible that HαCGRP$_{8-37}$ is a non-selective antagonist of separate CGRP and amylin receptors in vascular tissues.

1.1.9.2. Adrenomedullin

Intravenous administration of adrenomedullin to anaesthetized rats produces a potent and long-lasting hypotensive response (Kitamura *et al.*, 1993). It is approximately 10-fold less potent than HαCGRP as a vasodilator in the isolated rat mesenteric arterial bed. The extent to which adrenomedullin binds to CGRP receptors or its own specific receptors is not yet clearly defined. However, the vasodilatory response to

adrenomedullin in the isolated rat mesenteric arterial bed can be antagonized by HαCGRP$_{8-37}$ which suggests that it interacts, at least in part, with CGRP receptors (Nuki *et al.*, 1993). Adrenomedullin circulates in blood at relatively high concentrations (about 20 fmol/ml in plasma) and has been proposed as a hormone involved in the regulation of blood pressure.

1.1.10. Physiological and pathophysiological roles of CGRP

Intensive research over the 10 years since the discovery of CGRP has steadily unravelled its physiological and pathophysiological roles as a vasodilator. In some conditions, it is not unrealistic to predict that stable non-peptide agonists or antagonists of CGRP might be of therapeutic value. Indeed, CGRP itself has been the subject of clinical trials in man.

1.1.10.1. Neurogenic inflammation

The potent vasodilatory effect of CGRP in the skin suggests that it has a role in thermoregulation. Pathophysiologically, vasodilatation caused by intradermal CGRP potentiates the inflammatory oedema induced by a range of mediators of increased microvascular permeability in rabbit skin (Brain & Williams, 1985; Buckley *et al.*, 1991a). CGRP also potentiates substance P-induced oedema in rat skin (Brain & Williams, 1985; Gamse & Saria, 1985). Neutrophil accumulation is potentiated as a further consequence of its prolonged vasodilatory activity (Buckley *et al.*, 1991b). CGRP potentiates oedema formation induced by histamine in the rat knee joint (Cambridge & Brain, 1992) and active immunization with CGRP reduces the inflammatory response to adjuvant arthritis in the rat (Louis *et al.*, 1990). Thus CGRP may contribute to neurogenic inflammatory responses and a CGRP antagonist may have therapeutic potential as an anti-inflammatory agent.

1.1.10.2. Migraine

There is increasing evidence that CGRP may play a role in neurogenic inflammatory responses associated with migraine (reviewed by Moskowitz, 1992). During a migrainous headache, intracranial blood vessels may become distended and oedematous due to activation of trigeminal sensory nerve terminals and the subsequent release of neuropeptides. The neurogenic and vascular theories of the pathogenesis of migraine are controversial (Humphrey & Feniuk, 1991; Moskowitz, 1992). However, several findings implicate the involvement of CGRP. A substantial elevation of CGRP levels occurs in the external jugular venous blood of patients suffering from classic or common migraine, probably reflecting release from activated

sensory fibres (Goadsby *et al.*, 1990). Electrical stimulation of the trigeminal ganglion in rats leads to rapid increases in plasma CGRP levels in the superior sagittal sinus (Buzzi *et al.*, 1991). HαCGRP$_{8-37}$ has been successfully used to antagonize cerebral vasodilatory responses evoked by stimulation of the trigeminally-derived nasociliary nerve in the cat (Goadsby *et al.*, 1993). Thus CGRP may function as a vasodilatory neurotransmitter with pathophysiological roles in the trigeminovascular system.

1.1.10.3. Subarachnoid haemorrhage

In aneurysmal subarachnoid haemorrhage, CGRP may be released from the trigeminovascular system as a vasodilatory defence against vasospasm. The innervation of the cerebral circulation by CGRP-containing fibres is reduced after subarachnoid haemorrhage by up to 50% (Edvinsson *et al.*, 1990), and in patients who die after subarachnoid haemorrhage, there is depletion of CGRP immunoreactivity in brain vessels (Edvinsson *et al.*, 1991). A positive correlation (r=0.7) has been found between CGRP levels in the jugular blood and an index of vasospasm in patients with middle cerebral artery aneurysms (Juul *et al.*, 1990). These findings have prompted clinical trials of CGRP for the prevention of ischaemic deterioration after surgery for aneurysmal subarachnoid haemorrhage. However, clinical benefit has not been clearly demonstrated and there is a need for larger clinical trials to be performed (European CGRP in subarachnoid haemorrhage study group, 1992).

1.1.10.4. Raynaud's phenomenon

Selective vascular supersensitivity to CGRP has been demonstrated in the hands of patients with Raynaud's phenomenon (Shawket, *et al.*, 1989) and a significant reduction in the number of CGRP-immunoreactive neurons has been found in the digital skin of such patients (Bunker *et al.*, 1990). These findings suggest that a deficiency of CGRP is relevant to the pathogenesis of Raynaud's phenomenon. Indeed, Shawket *et al.* (1991) and Bunker *et al.* (1993) have demonstrated the therapeutic benefit of i.v. CGRP infusions in this condition.

1.1.10.5. Hypertension

In normal volunteers, assumption of the upright posture leads to a quick rise in plasma CGRP levels together with expected increases in plasma noradrenaline and aldosterone and plasma renin activity. Intravenous infusion of angiotensin II results in dose-dependent increases of plasma CGRP levels (Portaluppi *et al.*, 1993). These findings suggest that CGRP is released as a physiological response to changes in posture and vasomotor tone. However, there are conflicting reports on circulating

CGRP levels in hypertensive patients. Significantly lower plasma CGRP levels have been found in patients with severe hypertension (Edvinsson *et al.*, 1989a) and uncomplicated essential hypertension (Portaluppi *et al.*, 1992) compared with normotensive controls. Another study found no significant difference in serum CGRP levels between patients with untreated mild to moderate hypertension compared with age- and sex-matched normotensive controls (Schifter *et al.*, 1991). In contrast, Masuda *et al.* (1992) reported significantly higher plasma CGRP levels in patients with essential and secondary (primary aldosteronism and phaeochromocytoma) hypertension compared with normotensive control subjects. Moreover, a significant positive correlation between systolic and diastolic blood pressures and plasma CGRP levels was demonstrated.

The role of CGRP in the spontaneously hypertensive rat (SHR) model of human essential hypertension is also unclear. Neurogenic vasodilatation and CGRP-immunoreactivity release induced by perivascular nerve stimulation of the perfused mesenteric arterial bed is significantly decreased in SHRs compared with normotensive Wistar-Kyoto controls (Kawasaki *et al.*, 1990b). Both neuronal CGRP levels and CGRP mRNA levels are decreased in SHRs (Supowit *et al.*, 1993; Westlund *et al.*, 1991) with no apparent difference in vascular responses to CGRP (Ando *et al.*, 1990) compared with normotensive Wistar-Kyoto controls. On the other hand, Zaidi *et al.* (1991) found higher neurally-derived plasma CGRP levels in SHRs compared with normotensive controls. Thus, at present, it is difficult to reconcile the competing hypotheses of (1) a pathological impairment of the synthesis or release of CGRP in hypertension and (2) a physiological enhancement of CGRP release as mechanism to compensate for increased peripheral vascular resistance in hypertension.

1.1.10.6. Pregnancy and fluid overload

CGRP may be a physiologically important vasodilator during pregnancy. Plasma volume is expanded by about 40% but changes in peripheral vascular tone usually prevent an increase in blood pressure. Data from two studies show that plasma CGRP levels rise gradually during pregnancy to a peak (about 3-fold increase) in the ninth month and return to baseline 5 days post-partum (Stevenson *et al.*, 1986; Saggese *et al.*, 1990). The tissue content of CGRP in uterine arteries from pregnant women is significantly higher than in non-pregnant women (Nelson *et al.*, 1993a). Moreover, the sensitivity of the isolated uterine artery to CGRP is higher during pregnancy. These findings are consistent with suggestions that CGRP is in part responsible for changes in peripheral vascular tone in response to plasma volume expansion.

Plasma CGRP levels are also increased in other states of chronic volume expansion such as hepatic cirrhosis (Bendtsen *et al.*, 1991) and in acute fluid overload during haemodialysis (Odar-Cederlof *et al.* 1991). Plasma CGRP levels increases with the severity of cirrhosis and are strongly correlated with fluid excess during haemodialysis.

1.1.10.7. Congestive cardiac failure

Elevated circulating CGRP levels have been detected in congestive cardiac failure (reviewed by Preibisz, 1993). Intravenous infusion of CGRP in patients with severe congestive cardiac failure leads to increased cardiac output, renal blood flow and glomerular filtration rate while systemic and pulmonary vascular resistance are decreased (Gennari *et al.*, 1990; Shekhar *et al.*, 1991; Stevenson *et al.*, 1992). Although no tolerance develops during prolonged infusions, the therapeutic use of CGRP in this condition is limited by the rapid offset of the beneficial effects (within 30 minutes) on cessation of infusion. A selective, orally available, non-peptide CGRP agonist with a long duration of action may be therapeutically useful.

1.1.10.8. Myocardial ischaemia

The effects of CGRP on the coronary circulation has been studied in some detail on the premise that CGRP may regulate coronary tone and participate in reactive hyperaemia following myocardial ischaemia. Circulating CGRP levels are elevated following acute myocardial infarction (reviewed by Preibisz, 1993). Myocardial ischaemia leads to CGRP release from the isolated perfused guinea-pig heart (Franco-Cereceda *et al.*, 1987b) and CGRP-induced relaxation of sheep coronary artery rings is enhanced under simulated ischaemic conditions (Kwan *et al.*, 1990). Intracoronary infusion of CGRP in man leads to dilatation of both normal and artheromatous epicardial coronary arteries showing irregularity at angiography (Ludman *et al.*, 1991). However, studies in dogs suggest that CGRP does not have a significant role in the regulation of microvascular tone during acute myocardial ischaemia (Sekiguchi *et al.*, 1994). HαCGRP$_{8-37}$ blocked the vasodilatory effect of exogenous HαCGRP but had no effect on the diameters of coronary arterial microvessels during acute myocardial ischaemia (induced by occlusion of the left anterior descending coronary artery).

1.1.10.9. Sepsis

Decreased vascular resistance and increased cardiac output occur in the hyperdynamic state of sepsis. Joyce *et al.* (1990) reported that patients with pyrexia, elevated white

cell counts, bacterial septicaemia and hyperdynamic status had 7.5-fold higher plasma CGRP levels compared with normal volunteers; this finding suggests that CGRP may be a beneficial modulator of the hyperdynamic circulation during sepsis. On the other hand, studies in rats suggest that excessive stimulation of CGRP release by endotoxins could lead to septic shock. Administration of Gram-negative bacterial endotoxin to conscious rats led to a 22-fold increase in plasma CGRP levels followed by cardiovascular collapse (Wang *et al.*, 1992). The tachycardia and hypotension could be transiently reversed by i.v. administration of HαCGRP$_{8-37}$ (Huttemeier *et al.*, 1993).

1.1.10.10. Other possible roles

The above overview of the potential physiological importance of CGRP has been confined to the vasodilatory role of CGRP. Other possible roles of CGRP as a vasodilator include gastric mucosal protection (Holzer *et al.*, 1991; Lambrecht *et al.*, 1993) and the modulation of pulmonary hypertension following chronic hypoxia (Tjen-A-Looi *et al.*, 1992). The non-vasodilatory effects of CGRP are broad (Tables 1.1 and 1.2) and it must be recognized that CGRP may play diverse roles outside the cardiovascular system.

1.2. Monoclonal antibodies as pharmacological tools

1.2.1. Antibodies

Antibodies are host glycoproteins produced in response to the presence of foreign molecules in the body. Humoral immunity due to circulating antibodies in the γ-globulin fraction of plasma proteins is a major defence against bacterial infections. Antibodies are synthesized primarily by plasma cells, a terminally differentiated cell of the B-lymphocyte lineage.

Proteins which have the general structural features of antibodies, but do not have known antigen binding properties, are known as immunoglobulins. Five classes of immunoglobulin antibodies (IgG, IgM, IgE, IgA and IgD) are produced by the lymphocyte-plasma cell system. The major structural features of antibodies may be discussed by considering monomeric IgG which is the most abundant immunoglobulin in serum. The antibody molecule may be visualized as a symmetrical Y-shaped structure consisting of two identical polypeptide heavy chains and two identical polypeptide light chains. The glycosylated heavy chains are joined by disulphide bonds to each other, and each non-glycosylated light chain is joined by a disulphide bond to one heavy chain. There are five types of heavy chains, which

determine the class of immunoglobulin molecules, and two types of light chains (κ and λ). Over 95% of light chains are κ in the mouse and rat. The molecular weight of the heavy and light chains of IgG are approximately 55 kDa and 25 kDa respectively.

Each heavy and light chain is made up of several homology units of about 110 amino acids, which form globular regions termed domains. Each heavy chain comprises one variable (V_H) and three constant domains (C_H^1 to C_H^3), whereas light chains consist of a single variable (V_L) and a single constant domain (C_L). Within each variable domain lie 3 distinct hypervariable regions which form the majority of contact residues for the binding of the antibody to the antigen. The hypervariable regions are also known as complementary determining regions (CDRs). The two variable domains, V_H and V_L from the heavy and light chains respectively, are folded in such a way that brings the 6 hypervariable regions together to form an antigen-combining site. Each IgG molecule has two identical antigen-combining sites at the tip of the arms of the Y-like structure. The remarkable specificity of antibodies to the vast number of possible antigens is attributable to variability in both the length and amino acid composition of the hypervariable regions.

1.2.2. Monoclonal antibodies

Until 1975, all antibodies were obtained from the serum of immunized animals. The antibodies were polyclonal in nature and suffered from the disadvantage of batch-to-batch variation arising from intra- (between immunization) and inter-animal variability. The availability of polyclonal antibodies (PAbs) was limited by the blood volume and life span of the animal.

The publication of a method for the production of unlimited quantities of monospecific antibodies (monoclonal antibodies; MAbs) by Kohler & Milstein (1975) transformed the field of immunology and, before long, the new MAb technology was exploited in virtually all areas of biology and medicine. Kohler and Milstein reported the successful fusion of proliferating B-lymphocytes from mouse spleen and myeloma cells in culture. The resulting hybrid cells, called hybridomas, inherit the properties of antibody secretion from the parent B lymphocytes and immortality from the parent myeloma cells. Thus potentially unlimited quantities of MAbs may be obtained from cloned hybridomas. The principles and techniques of MAb production are discussed in Chapter 3.

1.2.3. Anti-peptide MAbs: immunoblockade

Immunoblockade refers to the use of antibodies to bind hormones or neurotransmitters in order to prevent their interaction with receptors and thus block

Correction (page 25, lines 35-36): "Greves, 1984" should read "Greaves, 1984"

their biological effects. The technique has its origins in passive immunization which involves the use of a specific antiserum to neutralize microbial toxins. Passive immunization was first reported in 1890 by Behring and Kitasato who demonstrated that serum from an animal actively immunized against diphtheria toxin could be used to neutralize even a fatal dose of the toxin in another animal (reviewed by Llewelyn *et al.*, 1992).

Examples of the use of immunoblockade to study the physiological role of a hormone can be found in the literature of the 1950's. Following prolonged treatment with insulin, sera from several animal species abolished the hyperglycaemic effects of insulin injected simultaneously into mice (Maloney & Coval, 1955). Armin *et al.* (1960a) showed that hyperglycaemia could be dose-dependently induced by single i.v. injections of an anti-insulin serum whilst prolonged i.v. infusion of the antiserum led to a diabetic syndrome in conscious rats (Armin *et al.*, 1960b). Nevertheless, investigations into the effect of anti-insulin antibodies on insulin receptor binding has continued at least into the 1980's (e.g., de Pirro *et al.*, 1980; Komori *et al.*, 1986).

Immunoblockade offers an alternative to the synthesis of receptor antagonists and their application to the study of the physiological role of endogenous substances. The application of anti-peptide antibodies to assess the physiological role of neuropeptides, as opposed to hormones, is relatively new. There is a lack of information on the optimum conditions required for immunoblockade. Despite the introduction of hybridoma technology, immunoblockade studies have generally involved the use of PAbs. The variable apparent specificity, affinity and binding capacity of PAbs create problems in the study of antibody-antigen interactions. MAbs of defined affinity are an advantage in the quantitative study of immunoblockade. The use of a MAb avoids the potential complication of studying the interaction of antigen with different populations of antibodies found in serum such as high affinity-low capacity and low affinity-high capacity antibody sites commonly encountered in anti-insulin serum (Goldman *et al.*, 1978). Hybridoma technology allows the selection of MAbs with the desired fine specificity, degree of cross-reaction, affinity and physical properties for the purpose of immunoblockade.

1.2.4. Anti-receptor MAbs: receptor antagonism

Immunoblockade with anti-peptide antibodies offers an alternative to the more conventional application of peptide or non-peptide receptor antagonists. However, antibodies could also be developed against receptors for use as receptor antagonists. The applications of MAbs to the study of receptor biology are diverse (reviewed by Greves, 1984). From a pharmacological viewpoint, anti-receptor MAbs would be

particularly useful as functional receptor antagonists, tools for receptor localization and probes for receptor heterogeneity.

Various strategies have been advocated for the development of MAbs against membrane receptors. The most direct route is to immunize animals with isolated receptor (Strosberg & Schreiber, 1984). Although classic, this procedure is applicable after only considerable progress in the purification of the receptor protein. Immunization with receptor-rich membranes or whole cells may be attempted when purified receptor is unavailable. If the receptor has been cloned or partially sequenced, a synthetic peptide corresponding to the desired portion of the receptor molecule may be used for immunization (e.g., Mestikawy *et al.*, 1990; Azmitia *et al.*, 1992).

Anti-receptor antibodies have also been generated by a technique known as the complementary peptide strategy. It has been postulated that peptide ligands and their receptors can each be encoded, at least in part, by complementary nucleotide sequences. Thus complementary synthetic peptides representing part of a receptor may be predicted from antisense mRNA that is complementary to the mRNA encoding a known peptide. This strategy was first used by Bost *et al.* (1985) who generated an antibody against a synthetic peptide complementary to corticotropin (ACTH) which recognized the adrenal cell ACTH receptor.

A popular strategy for the development of anti-receptor antibodies which does not require immunization with purified receptor is the anti-idiotypic approach. The strategy involves development of anti-ligand antibodies which are then used as surrogate receptors for immunization. This is followed by screening for anti-idiotypic antibodies, a subset of which will also recognize the receptor for the ligand. A one-step auto-anti-idiotypic approach which exploits the presence of a normally functioning idiotypic-anti-idiotypic network is a further, and potentially more effective, method for the generation of anti-receptor MAbs. The anti-idiotypic route to anti-receptor antibodies is reviewed in detail in Chapter 6.

1.3. Aims of the project

The review of the localization, release, receptor binding and biological effects of CGRP in Section 1.1 indicates that it is an endogenous substance with potential autonomic, metabolic, somatosensory, integrative and motor functions. However, the physiological importance of many of the biological effects attributed to CGRP remains to be demonstrated. No CGRP antagonists were available when the project began. Therefore the challenge was, firstly, to develop and validate the pharmacological tools required, and secondly, to use these tools effectively to the

demonstrate the physiological role of CGRP, with particular reference to vasodilatation.

The aim of the project was to investigate whether vasodilatory responses to CGRP released from perivascular sensory nerves could be blocked with anti-CGRP or anti-CGRP receptor MAbs. Thus the project was motivated by both an interest in the role of CGRP as an endogenous vasodilator and the application of MAbs as pharmacological tools for probing the physiological role of a neuropeptide. The main aim was translated into several specific objectives which were:-

1. to develop anti-CGRP MAbs for immunoblockade studies.
2. to develop anti-CGRP receptor MAbs which could be used as specific antagonists of CGRP.
3. to demonstrate the utility of immunoblockade as a pharmacological technique.
4. to demonstrate the neurotransmitter role of CGRP.
5. to demonstrate the role of CGRP as an endogenous vasodilator.

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CHAPTER 2

Introduction to immunoblockade: pharmacokinetic and pharmacodynamic considerations

2.1. Introduction

Immunoblockade may be described as the blockade of the effects of a biological mediator by inhibition of its binding to specific receptors with antibodies directed against the mediator. The process of immunoblockade is illustrated schematically for CGRP in Figure 2.1. Compared with the quantitative study of receptor antagonism, immunoblockade often appears to be more of an art than a science. Only one other research group has made efforts to examine the theoretical aspects of immunoblockade (Tilders *et al.*, 1990; van Oers & Tilders, 1991; van Oers *et al.*, 1992). The efficacy of *in vivo* immunoblockade depends on the distribution of an antibody to the site of action in sufficient concentration, the relative affinity of the antibody and receptor for the endogenous ligand, and the elimination of the antibody. The purpose of this chapter is to consider the pharmacology of immunoblockade in kinetic and dynamic terms.

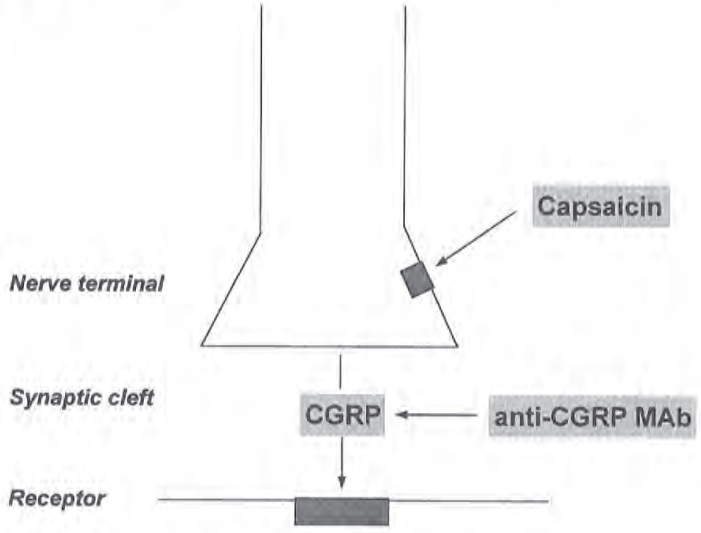

Figure 2.1: Schematic representation of the process of immunoblockade at a neuroeffector junction. Capsaicin acts on a prejunctional site to release CGRP from a sensory neuron terminal. Anti-CGRP MAb binds to CGRP in the synaptic cleft and inhibits receptor binding.

2.2. Pharmacokinetics

The time course of a drug in the body is dependent on the processes of absorption, distribution and elimination. The dosage regimen and the processes of absorption, distribution and elimination determine the concentration of a drug at its site of action and therefore the intensity of its effects as a function of time. Pharmacokinetics is concerned with the quantification of the relationship between dose and drug concentration over time. The pharmacokinetic properties of immunoglobulins, principally immunoglobulin G (IgG), are considered here. Differences between the pharmacokinetics of IgG, $F(ab')_2$ and Fab' fragments are highlighted[1].

2.2.1. Distribution of antibodies

The distribution of antibody molecules to the site of action is a prerequisite for immunoblockade. For the immunoblockade of CGRP, the site of action of antibodies is primarily the synaptic cleft which lies within the extravascular compartment. The capillary wall is the major barrier to the transport of macromolecules such as IgG to extravascular spaces (Garlick & Renkin, 1970; Arfors *et al.,* 1979). To gain access to extravascular targets, antibody molecules must pass through the endothelial lining of a capillary or postcapillary venule. The ability of intravascular solutes to cross the capillary barrier depends on molecular size. As illustrated in figure 2.2, capillary permeability falls steeply with increasing molecular size of solutes up to 4 nm, beyond which the decline is relatively gradual. During inflammation, however, high molecular weight proteins can escape from microvessels through gaps between adjacent endothelial cells (Arfors *et al.,* 1979).

It should be noted, however, that there are regional variations in the transcapillary transport of IgG. The discontinuous endothelial lining of the sinusoidal circulations of liver, spleen and bone marrow allows free passage of IgG. Thus it could be predicted, for example, that IgG will distribute rapidly to the extravascular spaces in the liver; immunoblockade of CGRP-induced changes in liver blood flow may be readily achieved. This is much less likely in the skin and muscle because the capillary permeability/surface area product (a measure of transcapillary movement) of IgG in the carcass is approximately 100 times lower than that in the liver (Covell *et al.,* 1986). On the basis of greater capillary permeability, Fab' fragments (molecular

[1]The fragmentation of MAbs is described in Chapter 4. Fab' fragments are produced from $F(ab')_2$ fragments by reduction followed by alkylation. Thus Fab' fragments are distinguished from Fab fragments which are univalent antigen binding fragments arising directly from digestion of IgG with papain. Fab' fragments are about 10% larger than Fab fragments but the small difference in molecular size is unlikely to be pharmacokinetically important. Fab' fragments will be considered in this chapter unless studies related directly to Fab fragments.

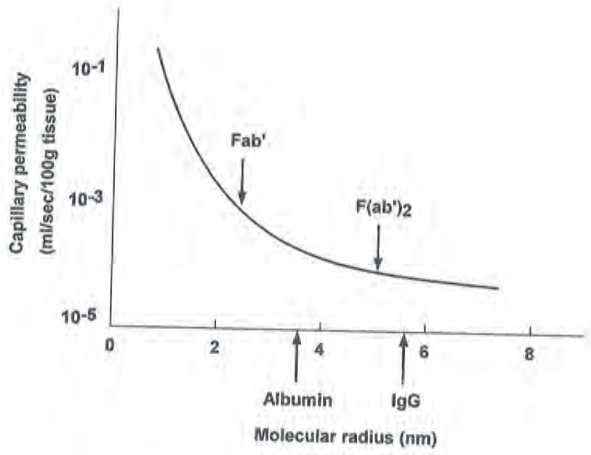

Figure 2.2: Relationship between molecular radius and permeability/surface area product which describes the movement of solutes from capillary plasma to interstitial fluid (Thomas *et al.*, 1989; Garlick & Renkin, 1970; Arfors *et al.*, 1979).

weight 50 kDa) may be predicted to be more accessible to the synaptic cleft within the interstitial space. Aubree-Lecat *et al.* (1993) have demonstrated by mathematical simulation that, for proteins above 100 kDa, the fraction of injected dose gaining access to the pharmacological target is principally limited by capillary permeability. When such a transport limitation exists, little benefit is gained by increasing the affinity of a macromolecule for its target or increasing the rate of uptake by the target.

IgG has a distribution volume that exceeds that of plasma plus interstitial fluid. However, non-specific whole IgG reside for the most part in the plasma compartment of the body (Covell *et al.*, 1986). IgG molecules do not bind to proteins within plasma but their distribution may be attributable in part to binding to Fc receptors present on many cell surfaces, particularly cells from lymphoid tissues and the reticuloendothelial system. Thus IgG may be retained in the liver, spleen and bone marrow through the interaction of its Fc fragment with Fc receptors on resident macrophages. Despite the similar size of Fc and Fab fragments, the pharmacokinetic profile of Fc fragment resembles that of IgG rather than Fab fragment (Arend & Silverblatt, 1975). The lack of Fc receptor interactions partially explains the pharmacokinetic differences between Fab fragments and IgG.

Covell *et al.* (1986) showed that non-specific homologous Fab' fragments have a larger total volume of distribution, distribute more rapidly into this volume, and produce a higher interstitial space to plasma concentration ratio compared with whole IgG. The mean steady-state volume of distribution of human Fab fragments in the rat

Correction (page 57, lines 2,23,26): "Llyod" should be spelt "Lloyd"

has been estimated as 0.43 l/kg (Pentel *et al.*, 1988) which is larger than extracellular volume in the rat (0.3 l/kg; Bianchi *et al.*, 1981). In the dog, Llyod & Smith (1978) reported an initial "distribution" half-life of 0.54 hour for sheep Fab fragments compared with 2.28 hours for IgG. This is in good agreement with a mean distribution half-life of homologous Fab in the rat of 0.25 hour compared with 2.2 hours for IgG (Arend & Silverblatt, 1975). Pentel *et al.* (1988) estimated a longer distribution half-life of 2.39 hours for human Fab fragments in the rat.

The distribution of $F(ab')_2$ fragments to capillary plasma, interstitial and cell-associated volumes and the rate of distribution into these volumes are comparable to those of IgG (Covell *et al.*, 1986).

2.2.2. Elimination of antibodies

IgG is metabolized primarily in the gut (72.8%) and the liver (20.5%). In contrast, Fab' fragment is metabolized principally by the kidney (73%) and the gut (22.9%) (Covell *et al.*, 1986). The elimination of Fab fragments is delayed in nephrectomized animals (Arend & Silverblatt, 1975) and is markedly impaired in patients with severe renal dysfunction (Ujhelyi *et al.*, 1993). Radioactive-labelled Fab fragments, but not IgG, may be autoradiographically detected in renal proximal tubule cells after i.v. administration (Arend & Silverblatt, 1975). Antibody-antigen complexes are removed from the circulation primarily through phagocytosis by macrophages.

Covell *et al.* (1986) estimated that the mean residence times of homologous non-specific IgG_1, $F(ab')_2$ fragment and Fab' fragment were 8.3 days, 0.5 days and 0.24 days respectively. Reported elimination half-lives of various IgG with specific binding properties range from 2.1 to 7.4 days (Llyod & Smith, 1978; Shockley *et al.*, 1992; Wawrzynczak *et al.*, 1992). The elimination half-life of non-specific human Fab fragments in rats (Pentel *et al.*, 1988) and sheep digoxin-specific Fab fragments in dogs (Llyod & Smith, 1978) have been reported as 16.3 and 17.1 hours respectively. The more rapid elimination of Fab' fragment leads to a lower number of cycles through the interstitial and cell-associated compartments compared with IgG, and therefore, fewer opportunities for binding (Covell *et al.*, 1986). This potential disadvantage may be overcome by the use of higher doses and repeated administration of Fab' fragment when necessary.

Kinetic parameters estimated in some studies should be interpreted cautiously because of inadequate blood sampling periods following antibody administration. For example, the pharmacokinetic parameters of IgG and Fab fragments estimated from blood or plasma concentrations observed up to 3 hours post-dose (Triguero *et al.*, 1991; Johnston *et al.*, 1988) are very different from those estimated from plasma or

serum concentrations observed up to 80 hours post-dose (Shockley *et al.*, 1992; Pentel *et al.*, 1988). The low volume of distribution of Fab fragment (0.046 l/kg) estimated by Johnston *et al.* (1988) is likely to reflect an initial distribution volume and the short half-life termed an "elimination half-life" (110 to 115 minutes) is more likely to reflect distribution as well as elimination. However, differences in pharmacokinetic parameters reported could also be due to differences in species from which antibodies were derived, the species to which the antibodies were administered and dose-dependent pharmacokinetics.

Repeated administration of heterologous IgG may elicit a strong anti-species immune response that could greatly increase IgG clearance. For example, Madon *et al.* (1991) found that the long-term effect of a sheep antiserum to rat growth hormone in rats was inversely related to its rate of elimination which, in turn, depended on the ability of the animal to mount an effective anti-sheep response. Heterologous Fab' fragment offers the advantage of lower immunogenicity compared with IgG.

2.2.3. Pharmacokinetics of different antibody classes

The various immunoglobulin classes differ in chemical characteristics such as size, charge, amino acid composition and carbohydrate content, and in their biological properties. Within each class, immunoglobulin subclasses exhibit further biological differences. Not surprisingly, the half-lives of immunoglobulins of different classes in serum are variable (Goding, 1986). However, studies which have formally compared the pharmacokinetics of purified immunoglobulins of defined class and subclass are lacking. Such comparisons are difficult because the pharmacokinetics of a particular antibody is likely to depend, in part, on its binding characteristics, the species it was derived from and the host species. Studying a series of mouse anti-melanoma MAbs in mice, Shockley *et al.* (1992) reported shorter terminal half-lives of approximately 85 hours for IgG_1 compared with 110 to 115 hours for IgG_{2a}.

The specific binding of a peptide by antibodies of different subclasses may have variable kinetic consequences *in vivo*. In the case of insulin, ^{125}I-insulin complexed to guinea pig IgG_2 is cleared significantly more rapidly in the rat compared with ^{125}I-insulin complexed to guinea pig IgG_1. This may be due to more rapid sequestration of IgG_2 in the liver through an interaction with Fc receptors on Kupffer cells (Arquila *et al.*, 1987).

2.2.4. Pharmacokinetics of CGRP

Limited information is available on the pharmacokinetics of CGRP. The most detailed pharmacokinetic experiments have been performed in the sheep by Braslis *et*

Correction (page 59, line 26):
"... it the concentration ..." should read "... it is the concentration ..."

al. (1988). The clearance of HαCGRP (standard error) was 22.6 (2.1) and 15.0 (1.7) ml/kg/min when HαCGRP was infused to steady-state at 1 and 5 pmol/kg/min respectively. The significantly lower clearance at the higher infusion rate suggests that CGRP metabolism may be saturable or that clearance may be altered by haemodynamic changes induced by higher doses. The decline of plasma HαCGRP was biexponential with an initial half-life of 3.6 minutes and a terminal half-life of 13.6 minutes. The liver and kidney were the major organs involved in the clearance of HαCGRP with extraction ratios of 0.14 and 0.25 respectively. However, the clearance of HαCGRP by the liver, kidney, gut, lung and brain accounted for only a third of total body clearance suggesting that more generalized metabolic systems are involved. Using high performance liquid chromatography, Braslis *et al.* (1988) assessed the CGRP species detected by their radioimmunoassay and found that the major immunoreactive peak (which comprised more than 85% of CGRP-immunoreactivity) co-eluted with HαCGRP.

The clearance of HαCGRP in sheep agrees well with the clearance (standard error) of HαCGRP and HβCGRP in man which has been reported as 24.5 (2.9) ml/min/kg and 24.9 (5.6) ml/min/kg respectively following infusion of 4.4 pmol/kg/min to steady-state (Beglinger *et al.*, 1991). Struthers *et al.* (1986) measured plasma HαCGRP levels over 20 minutes following cessation of a constant i.v. infusion in man and estimated a half-life of 9.7 minutes. The pharmacokinetics of Rα CGRP in man was studied by Kraenzlin *et al.* (1985) who observed a biexponential decline of RαCGRP levels with longer initial half-life (6.9 minutes) and terminal half-life (29.4 minutes) than HαCGRP in sheep. Consistent with a longer terminal half-life, a lower clearance of 11 ml/min/kg was estimated for RαCGRP in man.

Although the above studies have provided useful information on the global pharmacokinetics of CGRP, it the concentration of CGRP at the synaptic cleft which is most relevant to immunoblockade studies of CGRP. No specific information is available but it is known that the intra-synaptic concentration of a classical neurotransmitter such as noradrenaline is inversely related to the width of the synaptic cleft (Bevan & Su, 1974). The minimum width of autonomic neuromuscular clefts varies considerably in different tissues, from about 20 nm in the vas deferens to 2 μm in large elastic arteries (Burnstock, 1986). Thus the concentration of CGRP achieved in the synaptic cleft is likely to vary among different tissues. The concentration of antibody required for immunoblockade of endogenous CGRP should be optimized for a particular tissue. For immunoblockade to be observed, antibody binding sites must not be saturated or if saturated, transfer of antibody to the cleft must be sufficiently fast.

The width of synaptic clefts also affects the time required for a transmitter to traverse the cleft, and therefore, the signal transfer time. The time (t) it takes a randomly jumping particle to move a distance x in one specific direction increases with the square of distance. Einstein showed that:

$$t = \frac{x^2}{2D}$$

where D is the solute diffusion coefficient. For example, the time for a glucose molecule to diffuse across a distance of 100 nm, which is comparable to a tight vascular neuromuscular gap, is 5×10^{-6} second (Levick, 1991). The short time required for synaptic forms of signal transfer has led some to doubt that immunoblockade of a neurotransmitter could really occur (see "Pharmacodynamics"; Section 2.3.3.).

CGRP-immunoreactive nerves are found in the adventitia or adventitial-medial border of blood vessels. At least some vascular smooth muscle cells will be directly innervated and signal transfer is likely to be rapid. However, endothelium-dependent vasodilatation has also been reported and this implies relatively slow diffusion of CGRP through the media to the intima of blood vessels.

A further consideration that is relevant to chronic immunoblockade studies *in vivo* is whether blockade of a neuropeptide would promote positive feedback release such that the concentration of free neuropeptide is returned to normal. This pharmacokinetic question has not been addressed experimentally.

2.3. Pharmacodynamics

Pharmacodynamics is the study of the biochemical and physiological effects of drugs and their mechanisms of action. Defined as such, it is a broad subject that encompasses molecular mechanisms to the quantification of the relationship between drug concentration and the magnitude of drug effect.

2.3.1. Mechanisms of immunoblockade

The mechanism of immunoblockade is generally proposed to be an interference with signal transfer through the formation of an inactive immune complex between antibody and peptide during the intercellular journey of the peptide from the site of secretion to the site of action (Figure 2.1). Several assumptions that are implicit in this proposed mechanism of action should be critically examined. The binding of an

antibody to a peptide may not lead to inhibition of the biological activity of the peptide. Indeed, depending on the epitope on the peptide an antibody is directed against, potentiation of biological activity may occur; this has been reported, for example, for antibodies directed against corticotropin releasing factor, growth hormone and CGRP (Tilders *et al.*, 1990; Shaw *et al.*, 1992). Antibody binding may also potentiate or prolong the biological effect of a peptide by inhibiting its metabolic breakdown.

When an antiserum is used, it is possible that some of the observed effects may be due to an interaction between auto-anti-idiotypic antibodies and receptors (Chapter 7). There is also evidence that antibodies to neuropeptides can be internalized into specific neurons after local administration in the brain. Tilders *et al.* (1990) reviewed the evidence for the uptake of substance P, vasopressin and corticotropin releasing factor into neurons containing the respective peptides and suggested that the internalization of antibodies to peptide-containing cell bodies leads to inhibition of their secretory activity. Although the mechanisms of neuronal uptake of antibodies are unclear, antibody binding to peptides within neurons does challenge the assumption that immunoblockade always occurs through the interaction of antibody and peptide in liquid phase. Even if the neuronal uptake of native antibodies is not a general phenomenon, it may be induced by the cationization of antibodies. Triguero *et al.* (1989, 1991) have demonstrated that cationized antibodies retain antigen binding properties and exhibit enhanced uptake into cells.

2.3.2. Antibody-antigen interaction

The interaction between antibody (Ab) and antigen (Ag) and the antibody-antigen complex (AbAg) may be expressed as:

$$Ab + Ag \Leftrightarrow AbAg$$

The rate of complex formation = $k_1[Ab][Ag]$ and the rate of complex dissociation = $k_2[AbAg]$ where k_1 and k_2 are the on-rate and off-rate constants respectively. At equilibrium, the rate of complex formation = rate the of complex dissociation , and

$$k_1[Ab][Ag] = k_2[AbAg]$$

Therefore,

$$\frac{k_2}{k_1} = Kd = \frac{[Ab][Ag]}{[AbAg]}$$

where Kd is the equilibrium dissociation constant which is equal to the concentration of antibody producing half-maximal binding. The affinity[2] of Ab for Ag is described by the affinity constant (reciprocal of Kd).

Under the condition that the [Ab] is considerably higher than [Ag] (when most Ab is not involved in binding), the ratio of free over bound Ag will be primarily dependent on [Ab] and Kd:

$$\frac{[Ag]}{[AbAg]} = \frac{Kd}{[Ab]}$$

By increasing [Ab], the ratio of free to bound Ag declines proportionally. Thus the extent of immunoblockade may be assessed by examining the relative values of Kd and [Ab]. For example, when [Ab] is 100 times higher than Kd (i.e. 100Kd),

$$\frac{[Ag]}{[AbAg]} = \frac{1}{100} = 0.01$$

$$[Ag] = 0.01[AbAg]$$

Substituting for [Ag], it can be calculated that 99% of the antigen will be bound:

$$\text{Proportion bound} = \frac{[AbAg]}{[Ag]+[AbAg]} = \frac{1}{1.01} \times 100 = 99.0\%$$

2.3.3. Effect of antibody-ligand interaction on pharmacological response

The considerations above are limited to the assessment of the likelihood and extent of immunoblockade when antibody concentrations are considerably higher than antigen concentration. However, it is more informative to study the effect of an antibody over the entire concentration-response range of a ligand.

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[2]Affinity is a thermodynamic measurement of the strength of the non-covalent interaction between one site of the antibody and of the antigen. Avidity, on the other hand, is an operational term expressing the ability of an antibody preparation to bind antigens and depends, therefore, not only on affinity but also on multivalency and other non-specific factors. It is common that the multivalent IgM has an avidity (functional affinity) of 10^2 to 10^4 times higher than the affinity of isolated sites (Fab fragments).

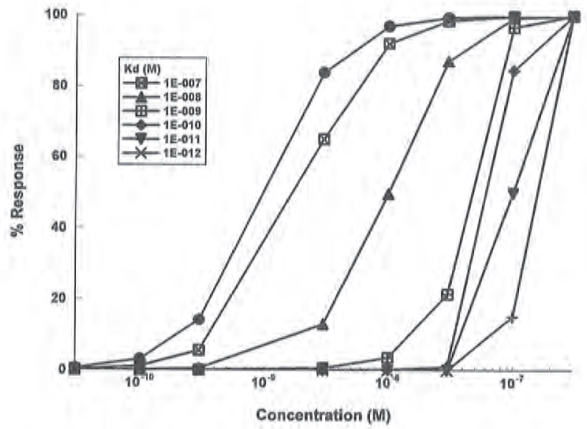

Figure 2.3: Effect of dissociation constant (Kd) of antibody-ligand binding on the concentration-response curve of the ligand. Pharmacological response was simulated with the logistic model using the following parameter values: $E_0 = 0$; $E_{max} = 100\%$; $EC_{50} = 1$ nM; N = 1.5. The concentration-response relationship in the absence of anti-ligand antibody is plotted with closed circles. Kd was varied from 10^{-7} to 10^{-12} M (1E-007 to 1E-012 M) with B_{max} fixed at 100nM.

Given the dissociation constant of antibody binding to ligand (Kd), the concentration of binding sites (B_{max}), and the total concentration of ligand (C), the concentration of ligand bound (B(C)) could be predicted from the following equilibrium binding model:

$$B(C) = \frac{B_{max} \times C}{Kd + C}$$

The unbound ligand concentration may be obtained by subtraction of bound from total ligand concentration. Assuming that only unbound ligand is active, the pharmacological effect (E) of ligand in the presence of anti-ligand antibody could be simulated with the sigmoidal E_{max} (logistic) model below (Holford & Sheiner, 1981):

$$E = E_0 + \frac{E_{max} \times C^N}{EC_{50}^N + C^N}$$

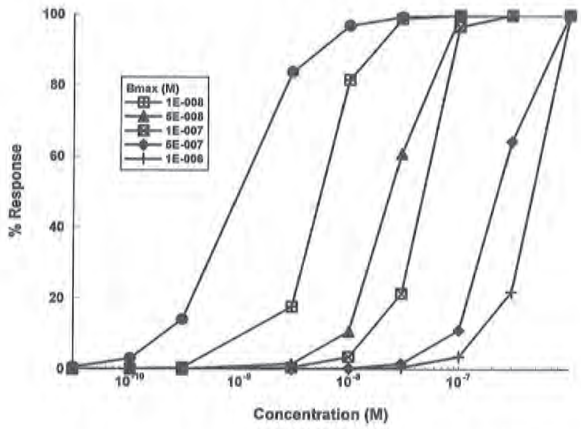

Figure 2.4: Effect of concentration of antibody binding sites (B_{max}) on the concentration-response curve of a drug. Pharmacological response was simulated with the logistic model using the following parameter values: $E_0 = 0$; $E_{max} = 100\%$; $EC_{50} = 1$ nM; $N = 1.5$. The concentration-response relationship in the absence of antibody directed against the drug is plotted with closed circles. B_{max} of the immunoblocking antibody was varied from 10^{-8} to 10^{-6} M (1E-008 to 1E-006 M) while Kd remained constant at 1nM.

where E_0 is the baseline effect, E_{max} the maximum effect, EC_{50} the concentration which produces half-maximal effect and N the steepness factor (Hill coefficient).

Changing the value of one parameter at a time, the influences of antibody Kd and B_{max} on pharmacological response were simulated for a range of concentrations commonly encountered in pharmacological experiments. Figures 2.3 and 2.4 shows the consequences of using antibodies of different binding affinities and different concentrations of a particular antibody respectively. Due to the non-linearity of the antibody binding and pharmacodynamic models, EC_{50} does not shift proportionally with changes in Kd and the shifts in concentration-response curves are non-parallel. The simulations clearly indicate that a high concentration of a high affinity (low Kd) antibody produces the greatest blockade of pharmacological response.

A major assumption of the above simulations is that equilibrium binding of antibody to ligand is achieved prior to the arrival of ligand at the receptor. This is most valid when the time for signal transfer is long, i.e. an antibody will have a high probability of achieving maximal binding to ligand prior to arrival of ligand at the receptor. van Oers & Tilders (1991) showed that the time to achieve maximal antibody binding is determined by the on-rate constant, Kd and the local antibody concentration. These authors performed simulations based on the theoretical framework they presented, and concluded that immunoblockade of signalling

processes that take less than 10 milliseconds is unlikely. They expressed doubt if peptidergic synaptic transmission could be blocked and suggested that most positive results on the immunoblockade of neuropeptides probably represent interference with non-synaptic transmission. However, Tilders and co-workers have not considered the time-course of receptor binding. The on- and off-rates of antibody and receptor binding span similar ranges. Provided that an antibody is distributed to the synaptic cleft, the antibody and receptor will compete for binding to neuropeptide released into the cleft. The amount of peptide bound to antibody relative to receptor (and therefore the extent of immunoblockade) will depend on the relative on- and off-rates of antibody and receptor binding and the concentrations of antibody and receptor.

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CHAPTER 3

Development of monoclonal antibodies against CGRP

3.1. Introduction

The clonal selection theory (Burnet, 1959) postulated that each mammalian B lymphocyte has the potential to make only one antibody of a particular specificity. It is this theory that provides the conceptual framework for the development of monoclonal antibodies (MAbs). The revolutionary impact of MAb technology began with the discovery that fusion of a B-lymphocyte with a myeloma cell yields a hybrid cell which has inherited the properties of monospecific antibody secretion and immortality from the parent cells (Kohler & Milstein, 1975). The production of MAbs may be considered as 5 sequential processes:- (1) immunization, (2) fusion, (3) screening for antibodies of interest, (4) cloning, and (5) expansion of hybridoma cell lines.

The generation of a strong immune response to an antigen of low molecular weight usually requires coupling of the antigen to an immunogenic carrier protein. When the antigen is a water-soluble protein, the use of an adjuvant is usually necessary to augment the immune response. The most commonly used adjuvants are Freund's complete adjuvant (a water-in-oil emulsion in which killed and dried *Mycobacterium tuberculosis* are suspended in oil phase) and Freund's incomplete adjuvant (with bacteria omitted). The above considerations are relevant to CGRP since it is a relatively small water-soluble molecule that is recognized as "self" by the animal. The success of the immunization schedule may be assessed by testing the serum of immunized animals for specific binding activity. Spleens from animals with positive antisera are chosen for fusion with myeloma cells.

The original successful fusions were achieved by the use of inactivated Sendai virus but polyethylene glycol (PEG) is now established as the fusing agent of choice. PEG is favoured because it is commercially available, and its use results in a higher fusion frequency and greater reproducibility. Treatment of spleen and myeloma cells with PEG results in fusion of membranes and the formation of binucleate cells called heterokaryons. The nuclei fuse at the next cell division, generating hybrid cells.

The key to the successful selection of hybrid cells is the use of myeloma cells which lack the salvage pathway enzyme hypoxanthine guanine ribosyltransferase (HGPRT) and the culture of cells in medium containing hypoxanthine, aminopterin and thymidine (HAT). When the main synthetic pathways are blocked by the folic acid analogue aminopterin, the cell must depend on the salvage pathway enzymes HGPRT and thymidine kinase. $HGPRT^-$ myeloma cells die in HAT medium because

both the main and salvage pathways are blocked. Fusion with $HGPRT^+$ spleen cells provides $HGPRT^-$ myeloma cells with the missing enzyme, and the resulting hybrid cells are able to grow in HAT medium. Unfused spleen cells die in culture after a few days.

A rapid, sensitive and specific assay is required for the selection of culture wells containing hybridomas secreting the desired antibodies and for the isolation of individual clones secreting monoclonal antibodies. Cloning by limiting dilution is generally the preferred method. Once cloned, hybridoma cells may be propagated in culture or grown as ascitic tumours in histocompatible animals.

The objective of the work described in this chapter was to develop MAbs against CGRP which could be used in immunoblockade studies.

3.2. Methods

3.2.1. Conjugation procedure

Materials	Supplier
RαCGRP	Peninsula
1-ethyl-3(3-dimethylaminopropyl)-carbodiimide HCl	Sigma
Freund's complete and incomplete adjuvant	Behringwerke AG, Germany
Bovine serum albumin	Sigma

RαCGRP was conjugated to bovine serum albumin (BSA) using a water-soluble carbodiimide according to the general procedure described by O'Shaughnessy (1982). The conjugation reaction requires the presence of amino and carboxyl groups (Bauminger & Wilchek, 1980). In general, the amino groups are derived from lysyl residues of the protein carrier and carboxyl groups contributed by the hapten (aspartate and glutamate in RαCGRP).

RαCGRP (0.5 mg; 130 nmol) and BSA (2.3 mg; 32.5 nmol) were dissolved in 325 µl of deionized water pH 7.0, and 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride (16.25 mg; 81.25 nmol) was added to this solution. The mixture was incubated overnight at 4°C, aliquoted, and stored at -70°C.

3.2.2. Immunization protocol

3.2.2.1. Preparation of antigen (RαCGRP:BSA conjugate) in Freund's adjuvant

Fifty µl of the RαCGRP:BSA conjugate was diluted to 625 µl with water and mixed with 1875 µl Freund's adjuvant (3 volumes of the diluted aqueous immunogen) to

obtain a water-in-oil emulsion according to the following procedure. The aqueous immunogen was drawn into a plastic luer lock syringe and Freund's adjuvant into another plastic luer lock syringe. The syringes were connected with a three way tap. The aqueous solution was injected into the oil and the mixture passed rapidly back and forth between the two syringes a few dozen times (Goding, 1986). A water-in-oil emulsion was confirmed by allowing drops of the mixture to fall on to the surface of water in a beaker and observing discrete globules from the second drop onwards.

3.2.2.2. Immunization schedule

Ten 5 to 6 week old female Balb/C mice were immunized. Each mouse was identified by marking the ear with an ear clipper. Freund's complete adjuvant was used for the priming dose and Freund's incomplete adjuvant used in the first and second booster injections. Two hundred µl of the mixture, containing 35 µg of the immunogen, was injected into each mouse by the intraperitoneal (i.p.) route. The first and second booster injections were given at 4-weekly intervals. A final aqueous injection was given 3 days prior to fusion.

3.2.2.3. Screening of serum for anti-CGRP antibodies

Blood was collected from mice 8 days following the second booster injection. Each mouse was placed in a restrainer and blood collected by lightly cutting a tail vein with a scalpel. Two untreated mice were bled to obtain control serum. Blood was left to clot at 4°C and serum separated by centrifugation at 11,000 rpm for 5 minutes in a Biofuge B (Haraeus) centrifuge. Sodium azide (final concentration in serum 0.05%) was added to serum samples to minimize microbiological degradation.

3.2.3. Enzyme-linked immunoadsorbent assay (ELISA)

Buffers and reagents used in ELISA

Phosphate buffered saline (PBS) pH 7.4

NaCl	0.14 M	8.00 g
KCl	2.7 mM	0.20 g
KH_2PO_4 (anhydrous)	1.5 mM	0.20 g
Na_2HPO_4 (anhydrous)	8.1 mM	1.15 g
Deionized water		to 1 litre

(Adjusted to pH 7.4 with sodium hydroxide if necessary)

Acetate-Citrate Buffer pH 6

Sodium acetate 1 M

Adjusted to pH 6 with citric acid 1 M

Tetramethylbenzidine (10 mg/ml in dimethylsulphoxide; ICN Flow)

Tween 20 (0.05% v/v; Sigma)

Horseradish peroxidase conjugated rabbit anti-mouse antibody
(Heavy and light chains; concentrate diluted 1 in 1000 for use; ICN Flow)

Substrate reagent

1 M acetate-citrate buffer pH 6	1 ml
Deionized water	9 ml
Tetramethylbenzidine	100 µl
6% hydrogen peroxide	10 µl

3.2.3.1. Development of indirect ELISA screening assay

A chequerboard ELISA was used to determine the optimum coating concentration of CGRP (the antigen) and the titre of mouse antiserum. The general procedures are described below. A 96-well microtitre plate was coated with 50 µl serial 1 in 2 dilutions of CGRP (starting from 40 µg/ml) down each column. Serial 1 in 2 dilutions of mouse antiserum (starting from a 1 in 100 dilution; 50 µl) was added along each row of the plate. One control column was not coated with antigen. Serum from a non-immune mouse was added to a control row. It was found that a CGRP coating solution of 1.25 µg/ml produced good signals. For convenience, 1.5 µg/ml (400 nM; 75 ng in 50 µl) CGRP solution was used to coat plates in all subsequent ELISAs.

3.2.3.2. Experimental procedures

Ninety-six well microtitre plates (Linbro EIA plus titration plates; Flow) were coated with 50 µl of 400 nM CGRP solution or PBS vehicle per well, sealed with film, and left overnight at 4°C. Further steps were performed at room temperature. Plates were washed three times with 300 µl PBS/Tween 20 0.05% between each step of the ELISA. Wells were incubated with 100 µl porcine gelatin 0.25% in PBS pH 7.4 (blocking step) for 2 hours (or overnight at 4°C). Fifty µl serum or hybridoma culture supernatant (3 to 4 drops from a Pasteur pipette) was added to each well and incubated for 2 hours. When appropriate, serial dilutions were made in PBS/Tween 20

0.05%/gelatin 0.25%. Controls used were normal mouse immunoglobulin (10 μg/ml), culture medium and Sp2 myeloma cell culture supernatant. Wells were incubated with 50 μl horseradish peroxidase-conjugated rabbit anti-mouse antibody (1 in 1000 in PBS/Tween 20 0.05%/gelatin 0.25%) for 1 hour. One hundred μl substrate reagent (0.1 M acetate-citrate buffer pH 6 containing 0.006% hydrogen peroxide and 100 μg/ml tetramethylbenzidine as chromogen [Bos *et al.*, 1981]) was added to each well. Positive wells developed a blue colour when 25 μl of 2 M sulphuric acid was added to give a yellow colour with greater intensity. Absorbance was measured at 450 nm using an ELISA plate reader (Titertek Multiskan Plus Mk II, Flow Laboratories).

3.2.4. Radioimmunoassay (RIA)

Assay buffer pH 7.4

Na_2HPO_4	7.1 g	50 mM
EDTA	3.72 g	10 mM
BSA	3 g	0.3%
Deionized water		to 1 litre

Radioimmunoassay (RIA) was performed as a secondary screening assay. The RIA incubation mixture consisted of 50 μl hybridoma supernatant, 50 μl 2-[^{125}I]-iodohistidyl^{10}-HαCGRP (40,000 counts per minute) and 400 μl assay buffer. Tissue culture medium, Sp2 myeloma culture supernatant, normal mouse immunoglobulin (10 μg/ml) and a blank without supernatant were used as controls. The assay was performed at 4°C with a 4 day incubation period. Bound and free CGRP were separated by charcoal precipitation. One dextran-coated charcoal tablet (Steranti Separex; Steranti Research) was stirred in 15 ml separation buffer (assay buffer containing 0.25% gelatin) for 30 minutes at 4°C. Two hundred and fifty μl of the dextran/charcoal suspension was added to each assay tube followed by centrifugation at 2000 *g* for 20 minutes (Centra-7R centrifuge, IEC). Supernatant and charcoal were separated and the pellet counted for 3 minutes in a Beckman Gamma 5500 counter.

3.2.5. Preparation of feeder layer cells

One day prior to the day of fusion, MRC-5 human lung diploid fibroblasts (Flow; Long *et al.*, 1986) were trypsinized (Trypsin-EDTA, Sigma), counted and diluted to approximately 5-8 x 10^4 cells/ml. The cells were irradiated in 25 cm^2 flasks with 10,000 rads of high energy X-ray from a 20 MeV linear accelerator set at 16 MeV

(Department of Radiotherapy, Addenbrooke's Hospital). One hundred µl per well of the irradiated MRC-5 cell suspension were dispensed into five 96-well plates (Costar; low evaporation plates).

3.2.6. Preparation of myeloma cells

NSO (Flow; Kearney *et al.*, 1979) and Sp2 (Flow; Shulman *et al.*, 1978) myeloma cell lines were used in fusions. Both cell lines are non-producers of immunoglobulin heavy and light chains. Myeloma cells were thawed at least 2 weeks before fusion and grown in two 75 cm^2 flasks. Cells were fed daily for at least 3 days prior to the day of fusion. Both NSO and Sp2 cells grew well in Dulbecco's modified Eagles medium containing 2% foetal calf serum (2FD). Myeloma cells in stationary culture grow at approximately 3 x 10^5 cells/ml. Therefore two 75 cm^2 flasks (30 ml each) could be assumed to yield approximately 1.8 x 10^7 cells. Fusion was only performed if myeloma cell viability (measured by dye exclusion) was greater than 90%.

3.2.7. Fusion procedure

Equipment

1 x tea strainer/sieve (sterilized by autoclaving)
1 x large petri dish
2 x 100 mm petri dishes
1 x 5 ml syringe plunger
3 x universals containing 10 ml SFD (on ice)
3 x 50 ml tubes
3 x 10 ml sterilin tubes
1 x 10 ml sterilin tube containing 10ml SFD
150 ml SFD
2 x HAT in 10FD (50 ml/fusion)
5 x 96-well plates coated with MRC-5 feeder cells
Pipette tips (200 µl)
1, 10 and 25 ml sterile disposable pipettes
Plugged and unplugged sterile Pasteur pipettes
1 reagent trough
Improved Neubauer haemocytometer
2 pairs of sterilized scissors and forceps for dissection
2 pairs forceps (in laminar flow cabinet)
1 x universal with ethanol (for dissection)

1 x 2 ml syringe with needle

1 x 500 ml beaker with ice

1 washbottle containing 70% ethanol

Sterile consummables were generally obtained from suppliers. Other equipment was sterilized either in a Gallenkamp hotbox oven (160°C for 2 hours) or in a Denley autoclave (121°C for 20 minutes).

Materials	**Supplier**
Foetal calf serum	GlobePharm
Dulbecco's modified Eagles medium (DMEM) with 3.70 g/L $NaHCO_3$, with phenol red as pH indicator, without glutamine	Flow
Polyethylene glycol (PEG 1500; fusion tested); 1 ml/ fusion	BCL
Hypoxanthine (50x concentrate; 5 mM)	Flow
Aminopterin (50x concentrate; 2×10^{-5} M)	Flow
Thymidine (50x concentrate; 8×10^{-4} M)	Flow
L-glutamine (200 mM stock)	Flow
Penicillin (50,000 iu/ml stock)	Flow
Streptomycin (5000 µg/ml stock)	Flow
Amphotericin B (250 µg/ml stock)	Flow
Trypan blue	Sigma

Stock solutions (5 ml) of penicillin and streptomycin, amphotericin B and L-glutamine were added to 500 ml culture medium just before use.

3.2.7.1. Experimental procedures

The equipment above were assembled in the tissue culture laboratory and a mouse obtained from the animal house. Polyethylene glycol (PEG) and culture medium were allowed to warm up to room temperature. Three 10 ml aliquots of serum-free Dulbecco's modified Eagles medium (SFD) were placed in ice. Hypoxanthine, aminopterin and thymidine mixture (HAT) was thawed and 10% foetal calf serum in Dulbecco's modified Eagles medium (10FD) prepared.

The mouse was killed by cervical dislocation, placed in a large petri dish and soaked with 70% ethanol. The spleen was carefully removed. (Scissors and forceps were flamed before use. One pair of scissors and forceps were used to make incision into skin and a second pair of scissors and forceps used to make incision into the peritoneum and to remove spleen.) The spleen was placed in a 100 mm petri dish and transferred to a vertical-flow laminar flow cabinet (Gelaire BSB4). The mouse was removed from the laboratory. Hands were thoroughly washed and disinfected prior to further manipulations.

The spleen was washed twice by immersing in two 10 ml aliquots of ice-cold SFD and transferred to a tea strainer resting on a petri dish. Five ml cold SFD was added to the tea strainer and the spleen was pushed through the strainer using a 5 ml syringe plunger. Spleen cells were transferred to a 10 ml tube. The above procedure was repeated with another 5 ml cold SFD. The remaining cells were transferred to the 10 ml tube and cells passed up and down a Pasteur pipette several times to ensure adequate suspension. Clumps were allowed to settle for 3 to 5 minutes.

Myeloma cells were harvested from flasks using a 25 ml pipette and transferred to 50 ml tubes. Spleen cells were transferred to another tube with a Pasteur pipette. Care was taken not to take up any clumps. The spleen and myeloma cells were centrifuged at 400 *g* (1500 rpm; Denley centrifuge) for 5 minutes in the same spin. Supernatant was removed and spleen and myeloma cells resuspended in 10 ml SFD. Myeloma cells were counted (1×10^7 cells required total) using an improved Neubauer haemocytometer under a Nikon TMS inverted phase contrast microscope. The cell suspension was diluted 9:1 with 1% trypan blue in 0.9% saline to assess cell viability if required.

Spleen cells were added to myeloma cells in a ratio of 10 to 1 (10^8 spleen cells to 10^7 myeloma cells). The mouse spleen was assumed to produce 10^8 splenocytes in order to save time during the fusion. The cell mixture was made up to 50 ml with SFD, mixed well, and centrifuged at 400 *g* for 5 minutes. Supernatant was removed with the pellet left as dry as possible. The pellet was tapped gently and 1 ml PEG added from a 1 ml pipette over 1 minute with gentle and continuous stirring with the pipette tip. Immediately after the addition of PEG, 1ml SFD was added over 1 minute (very slowly initially), followed by 1 ml additions twice over 30 second intervals, and then at 15 second intervals with continuous stirring until 10 ml had been added. A further 15 ml SFD was added slowly, with continuous stirring. The mixture was centrifuged for 5 minutes at 400 *g*. Fifty ml 2xHAT in 10FD was prepared whilst cells were being centrifuged. The supernatant was removed. The pellet was tapped gently and cells resuspended in 50 ml of 2xHAT (for five 96-well plates). By the use of a multi-channel pipette, 100 µl of the cell suspension was transferred to each well of five 96-well plates containing MRC-5 feeder layer cells. The 96-well plates were kept in a humidified incubator (IR1500 5% CO_2, 37°C; Flow).

3.2.8. Post-fusion management

Plates were left undisturbed for 4 to 5 days after the day of fusion. Cells were fed after 4 to 5 days, and again after 7 to 10 days, with 1xHAT in 10FD. (Half the volume of

medium was removed from each well using a Pasteur pipette attached to vacuum line and replaced with fresh medium). Feeding was performed at least 3 times before screening to ensure that any antibodies initially secreted by unfused splenocytes were adequately diluted out to avoid false positive results in the screening assay. Hybridoma cells were cultured with HAT for a week or longer, and then with hypoxanthine and thymidine (HT) for at least 1 week. Positive cultures were duplicated, fed with separate medium (HT/10FD) to minimize contamination risks, and expanded in 24-well plates. The original fusion plates were kept for up to 6 weeks with feeding (HAT/10FD) at 7 to 10 day intervals until cell lines were frozen. Supernatants were screened and cell lines frozen and cloned as soon as possible after fusion.

3.2.9. Screening of supernatants

Supernatants from fusions were screened by ELISA (Section 3.2.3) when culture supernatants were turning yellow (acidic) and confluent hybridoma cells were visible under the microscope. After the blocking step, 3 to 4 drops of tissue culture supernatant were added from a Pasteur pipette to microtitre-plate wells coated with RαCGRP or PBS vehicle. Non-immune mouse immunoglobulin (10 μg/ml; Sigma), myeloma culture supernatant and fresh tissue culture medium were added to control wells.

3.2.10. Selection of positive hybridoma cells for cloning

It is important to clone hybridoma cells as soon as possible after positive wells have been identified to reduce the risk of overgrowth by non-producer cells. However, it was impractical to clone cells from the large number of positive wells identified in one of the fusions. Therefore further screening tests were performed as soon as possible to identify supernatants with the most desirable properties. Further assays included ELISAs to assess relative affinity and selectivity for different forms of CGRP, RIA and receptor binding inhibition assays.

3.2.11. Cloning by limiting dilution

MRC-5 feeder layer cells were prepared as described for the fusion procedure (100 μl per well; 5-8 x 10^8 cells/ml) at least 3 days before cloning. Hybridoma cells were maintained in exponential growth by appropriate feeding.

Cells were resuspended thoroughly with a Pasteur pipette and 0.5 to 1 ml

transferred to a 10 ml tube or universal. Trypan blue 1% solution was diluted 1 in 10 in cell suspension and viable cells counted. Cloning was postponed if there were fewer than 10^5 cells/ml. Cells were diluted 1 in 100 (100 µl + 10 ml HT/20FD). This was followed by serial dilutions in HT/20FD to give cell suspensions of 30 cells/ml (3 cells/well), 10 cells/ml and 3 cells/ml. One hundred µl of the diluted cell suspensions were dispensed into 96-well plates containing MRC-5 feeder cells to give 3 (half plate), 1 (half plate) and 0.3 cells per well (one plate).

Supernatants from wells with discrete clones from the 0.3 cells/well plate were screened by indirect ELISA. Cell lines were cloned twice in order to increase the probability of monoclonality.

3.2.12. Cryopreservation of hybridoma cells

Cell lines were fed regularly for several days to ensure that they were in good condition. Cells from each well of a 24-well plate were resuspended with a Pasteur pipette, transferred to sterile 10 ml tubes, and centrifuged at 400 *g* (1500 rpm; Denley centrifuge) for 5 minutes. Supernatant was removed and cells resuspended in 1 ml freezing medium (10% dimethylsulphoxide [ACS specification; Sigma] in foetal calf serum). The cell suspension was aliquoted to a 1 ml cryovial (Nunc). Cryovials were placed in a polystyrene box and frozen at -70°C for at least 24 hours. The cryovials were then stored in coded racks in a liquid nitrogen container (BT55, L'air Liquide, France). Records of the identity and location of frozen cell lines were kept in a computer database.

3.2.13. Thawing of cryopreserved cells

Cryovials were quickly removed from liquid nitrogen storage and placed in a 37°C water bath (Grant W28). Thawed cells were diluted with 5 ml 20FD (warmed to 37° C) as soon as possible and centrifuged for 5 minutes at 400 *g*. The supernatant was aspirated and discarded. The pellet was resuspended in 2 ml 20FD for seeding into one well of a 24-well plate containing MRC-5 feeder cells (0.5 ml; 5-8 x 10^8 cells/ml). Feeder cells were used since some cell lines were not successfully thawed and cultured in the absence of feeder cells. Alternatively, 20% MRC-5 feeder cell-conditioned medium in 20FD was successfully used for cell culture.

3.2.14. Cryopreservation of spleen cells

Spleens from positive mice which were not used for immediate fusion were frozen 3

days after a final booster injection of conjugated CGRP in aqueous solution. Spleen cells were resuspended at approximately 2 x 10^7 cells/ml in 5 ml freezing medium and frozen in 0.5 to 1 ml aliquots, as described in Section 3.2.12.

3.2.15. Bulk production of MAbs *in vivo*

MAbs may be produced in bulk by growing hybridoma cells in culture or as ascitic tumours in histocompatible animals (Galfre & Milstein, 1981). Large volumes of cell culture supernatants may be obtained at a concentration range of 2 to 20 µg/ml. However, more concentrated solutions were required for pharmacological studies and this was most readily achieved by growing cell lines *in vivo* and harvesting the resulting ascites with antibody concentration in the range of 2 to 20 mg/ml. There was another reason for the need to produce some MAbs *in vivo*. Some hybridomas would not grow in culture in the absence of feeder layer cells. Bulk production in roller culture was therefore not possible.

Balb/C mice were given a 100 µl i.p. injection of pristane (2,6,10,14-tetramethyl-pentadecane; Sigma) at least 7 days prior to i.p. injection of hybridoma cells. Hybridomas were in exponential growth when they were harvested, centrifuged (1500 rpm for 10 minutes; Denley centrifuge), and resuspended in normal saline. About 10^6 to 10^7 cells in 500 µl normal saline were injected into each animal i.p. using a 21 gauge needle. Mice were monitored daily for abdominal distension. Abdominal distension was apparent from 3 days post-injection, and ascites was generally tapped a week to two weeks post-injection. Ascites fluid was centrifuged at 2000 *g* for 10 minutes (Centra-7R, IEC; 3000 rpm), supernatant recovered, and activity checked by indirect ELISA. Sodium azide (10 mM) was added to ascites as a preservative. Ascites fluid was stored at 4°C for up to 1 month and at -70°C for longer periods.

3.2.16. Bulk production of MAbs *in vitro*

When C4.19 and C4.6 cell lines had been cloned twice (see "Results"), larger volumes of supernatant were obtained by culture of hybridoma cells in a 24-well plate, 25 cm^2 flasks, and eventually in 75 cm^2 flasks when 200 ml of supernatant was obtained after 3 weeks. The cell lines could be expanded further in roller culture if desired.

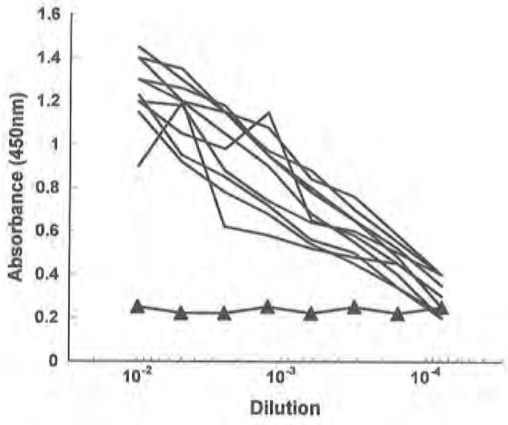

Figure 3.1: Binding of serum from mice immunized with RαCGRP to RαCGRP by indirect ELISA. All 10 mice were positive for anti-CGRP antibodies. Two-fold dilutions were made, starting from a serum dilution of 1 in 100. Symbols have been omitted for clarity except for triangles which show the lack of specific binding by serum from a non-immunized mouse.

3.3. Results

3.3.1. Immunizations

Following the second booster injection, all 10 mice immunized with RαCGRP were found to be positive in the ELISA for antibodies to CGRP with high titre whilst all controls were negative (Figure 3.1). Some of the antisera were also found to inhibit the binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to rat liver membranes in a receptor binding assay (described in Chapter 5). Mice whose serum was active in the binding inhibition assay (Figure 3.2) were chosen for fusions.

3.3.2. Fusions

Two fusions were performed with different results. The first fusion was performed using NSO myeloma cells. Supernatant from only one well was found to be positive by ELISA. The second fusion, performed using Sp2 myeloma cells, was highly successful. Hybridoma cells were detected microscopically in every well of the 96-well plates and supernatants from 100 wells were found to be strongly positive by ELISA. Positive cell lines were transferred to 24-well plates and frozen as soon as possible.

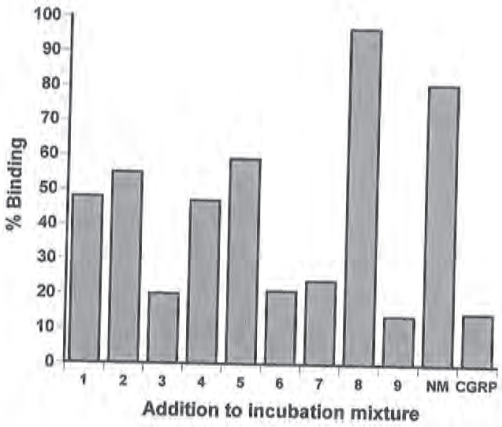

Figure 3.2: Effect of serum, diluted 1 in 100, from mice (1 to 9) immunized with RαCGRP on the binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to rat liver membrane preparation. Serum from a non-immunized mouse (NM) and excess RαCGRP (1 μM) were used as controls. Binding was expressed as a percentage of total binding in the presence of buffer alone. Values are the mean of two observations.

3.3.3. Cloning of selected cell lines

Due to the large number of positive supernatants, three selection criteria was applied in the choice of cell lines for cloning: (1) stability of the cell lines, (2) relative affinity of the antibodies for CGRP, and (3) cross-reactivity with different species of CGRP. An assumption of these selection procedures is that the fusion products had been plated sufficiently sparsely to give a high probability of monoclonality at the outset.

Sixty-four of the 100 cell lines expanded in 24-well plates were found to be positive when re-screened by ELISA, and these hybridomas were considered the more stable cell lines. Figure 3.3 shows that there were apparent differences in the affinity of antibodies from different hybridoma lines. The validity of this simple method of determining relative affinity (van Heyningen *et al.*, 1983) depends on prior knowledge that the variation of immunoglobulin concentration in supernatants from confluent hybridomas is generally less than ten-fold. The supernatants also demonstrated different cross-reactivities with the different forms of rat and human CGRP (Figure 3.4). Six cell lines were chosen for cloning on the basis of high relative affinity and selectivity for different forms of CGRP (Table 3.1).

Two further criteria were applied before cell lines were recloned. Until now, supernatants had been screened with an ELISA in which the antigen was immobilized

Table 3.1: Summary of first cloning results

Cell line	Number of wells with growth		
	0.3 cell/well	*1 cell/well*	*3 cells/well*
C4.63	13/96	12/48	30/48
C4.56	19/96	34/48	45/48
C4.19	18/96	26/48	45/48
C4.22	16/96	19/48	36/48
C4.5	17/96	29/48	43/48
C4.6	14/96	22/48	38/48

on solid phase. It is known that some antibodies which are active in an ELISA do not bind to antigen in liquid phase, for example, in a RIA. Since it was essential that the MAbs developed could bind to CGRP in liquid phase in immunoblockade experiments, supernatants were tested in a RIA and a receptor binding inhibition assay (described in Chapter 5). Supernatants from hybridoma lines 6 and 19 were strongly active in the RIA (Figure 3.5) and receptor binding assay (Figure 3.6). Thus the cell lines 6 and 19 were cloned again (Table 3.2). The MAbs were coded C4.6 and C4.19.

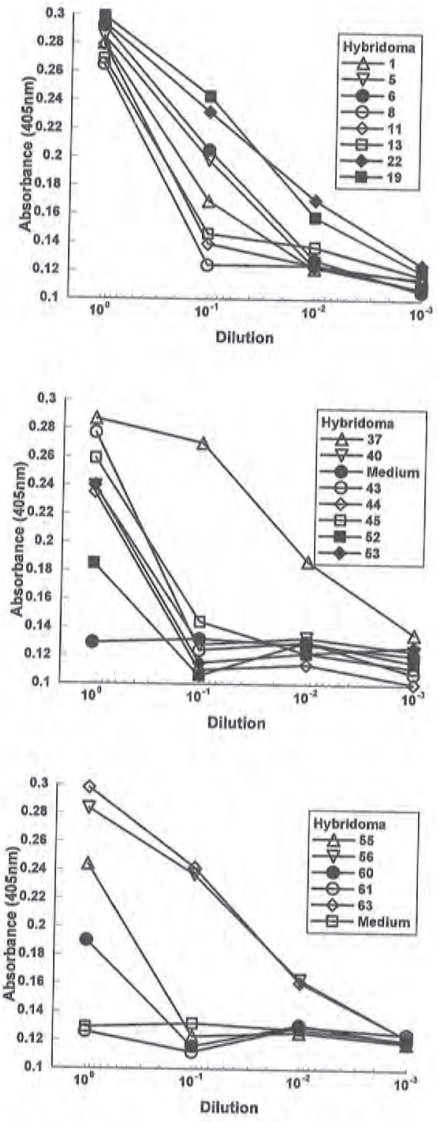

Figure 3.3: Relative affinity of hybridoma supernatants for RαCGRP by indirect ELISA. Tissue culture medium (10FD) was used as a control. Values plotted are single observations.

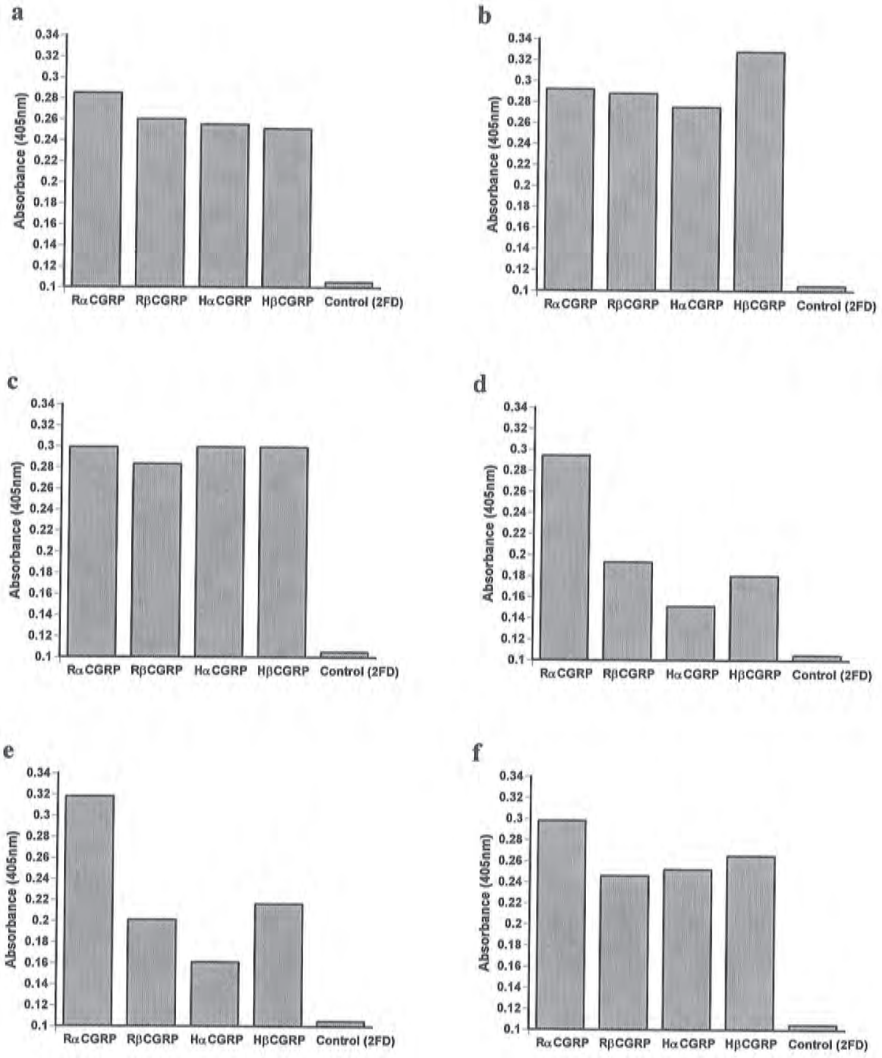

Figure 3.4: Binding of hybridoma supernatants C4.5 (a), C4.6 (b), C4.19 (c) C4.22 (d), C4.56 (e) and C4.63 (f) to different forms of rat and human CGRP by indirect ELISA. Values plotted are single observations.

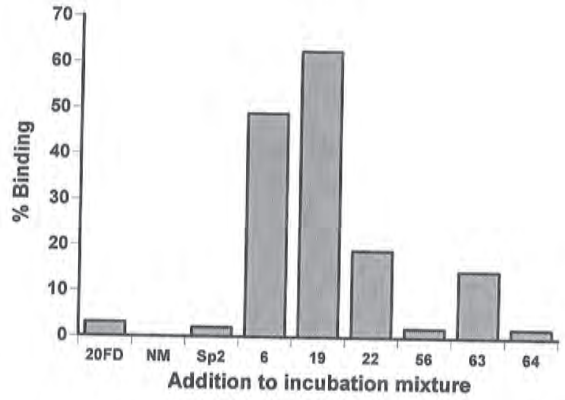

Figure 3.5: Binding of hybridoma supernatants 6, 19, 22, 56, 63 and 64 to 2-[^{125}I]-iodohistidyl^{10}-HαCGRP by RIA. Controls were culture supernatant (20FD), normal mouse immunoglobulin at 10 μg/ml (NM) and Sp2 myeloma cell supernatant. Binding was expressed as percentage of specific binding in the presence of buffer alone. Values are the mean of two observations.

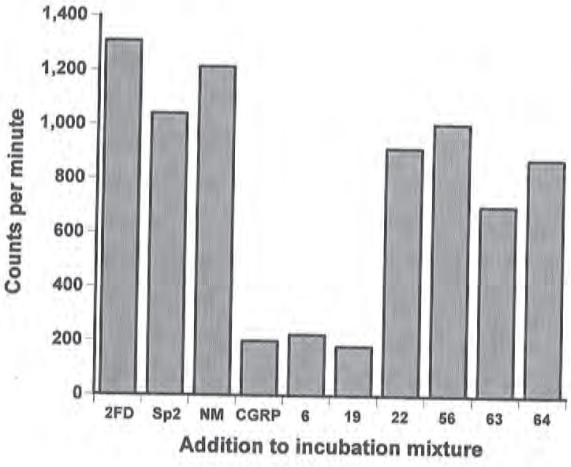

Figure 3.6: Effect of hybridoma supernatants 6, 19, 22, 56, 63 and 64 on the binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to rat liver membrane preparation. Controls were culture supernatant (2FD), Sp2 myeloma cell supernatant, normal mouse immunoglobulin at 10 μg/ml (NM) and excess RαCGRP (1 μM; non-specific binding). Values are the mean of two observations.

Table 3.2: Summary of second cloning results

Cell line	No. of wells with growth		
	0.3 cell/well	*1 cell/well*	*3 cells/well*
C4.6	8/96	13/48	27/48
C4.19	9/96	11/48	30/48

3.4. Discussion

The present work has led to the successful production of two MAbs (C4.6 and C4.19) which bind to CGRP by ELISA and RIA, and inhibit CGRP binding in a receptor binding assay. The MAbs have broad cross-reactivity with different forms of CGRP as assessed by indirect ELISA. The binding characteristics of MAbs C4.19 and C4.6 suggested that they would be promising candidates as immunoblocking agents.

Since the original report by Kohler & Milstein (1975), the procedure for MAb production has been modified by many groups. Because few formal controlled comparisons have been reported, it was difficult to select the most appropriate protocol from the literature. Nevertheless, it is possible to identify some factors which have most probably contributed to the successful production of anti-CGRP MAbs in this study.

Spleens from mice which had strongly positive serum in both ELISA and receptor binding assay were chosen for fusions. Discrepancies in the potency of different antisera in the receptor binding assay and ELISA could be accounted for by variable titres of antibodies which could inhibit receptor binding by binding to the active site of CGRP. Alternatively, CGRP receptor binding could be displaced by anti-CGRP receptor antibodies arising from a normally functioning anti-idiotypic network; this hypothesis is the basis of the auto-anti-idiotypic approach to anti-receptor antibodies (Chapter 6).

The myeloma cell lines used in this study were non-producers of immunoglobulin heavy and light chains. As a general rule, non-producer myelomas are the best choice since the hybrids will express only the antibody heavy and light chains of the parental spleen. Greater success was achieved with the Sp2 than the NS0 cell line in this study despite claims by others of fastidious growth characteristics and low fusion frequency with the Sp2 cell line (Goding, 1986). Whatever the choice of myeloma, one of the most important factors for the successful derivation of hybrids is the way in which the myeloma culture has been maintained prior to fusion. The aim is

Correction (page 87, line 14):
"The protocol followed ..." should read "The protocol which was followed ..."

to maintain exponential growth for as long as possible. More than a week of exponential growth is recommended (Galfre & Milstein, 1981).

The choice of foetal calf serum is critical since sera from different sources vary greatly. The batch of foetal calf serum used in the current work was tested for capability to support the growth of myeloma cells at 1 cell per well prior to use in fusion. The important variables in PEG-induced fusion are concentration, purity, pH and duration of exposure to PEG. The PEG used for the fusions described here had been tested for fusion capability by the supplier. Fusion frequency is reported to be highest at pH 8.0 (Westerwoudt, 1986). The duration of exposure to PEG may be critical since both fusion frequency and undesirable toxicity increase with time of exposure. Likewise, there is a trade-off between low concentrations (<30%) which give poor fusion frequency and high concentrations (>50%) which result in overwhelming toxicity. PEG may be tolerated for longer times at lower concentrations. The protocol followed attempted to exploit understanding of the above factors by the use of a high concentration of PEG initially, followed by gradual dilution over time.

Fusion products were plated into 96-well plates since monoclonality from the outset would be more likely compared with the use of 24-well plates. Fresh medium and supplements were always used for fusion and the early post-fusion stages of cell culture. Since every tissue culture manipulation increases the risk of contamination, manipulations were kept to a minimum.

The use of cell feeder layers improves fusion frequency and cloning efficiency. The growth characteristics of cells *in vitro* are influenced by the number of cells present in culture. Feeder cells are presumed to condition the medium and optimize the environment for hybridoma survival and growth. The use of feeder layers may be critical when cells are plated sparsely after fusion and during cloning. MRC-5 diploid fibroblasts have been clearly shown to be superior to a variety of other feeder layer cells (Long *et al.*, 1986).

Production of the MAbs was facilitated by the rapid, sensitive and specific ELISA screening assay. Porcine gelatin was used as the blocking agent in the ELISA instead of BSA because the mice were immunized with CGRP conjugated to BSA. The assessment of relative affinity and cross-reactivity by ELISA and secondary screening with the RIA and receptor binding assay provided a rational basis for the identification of hybrids for cloning and expansion. Screening procedures must be oriented as closely as possible towards the final application of the MAbs. The detection of immobilized CGRP in an ELISA was not the end point of the current work. Since the MAbs were being produced for immunoblockade studies, binding characteristics in liquid phase were assessed by RIA and the potential for

immunoblockade assessed by a simple receptor binding assay. The results of the RIA and receptor binding assay were consistent: only supernatants from hybrids C4.6 and C4.19 which were positive in the RIA could inhibit CGRP receptor binding.

In order to reduce the risk of overgrowth by non-producer cells and to ensure that the antibodies were truly monoclonal, cloning was performed as soon as supernatants with the desired properties were identified. The statistics of limiting dilution have been considered theoretically and verified experimentally (Coller & Coller, 1986). If cells are grown in small numbers, the fraction of wells with growth should follow the Poisson distribution. The Poisson equation assumes a random distribution of elements in the suspension being sampled. Thus it is important that the cell suspension is thoroughly mixed before performing limiting dilution cloning. Using the equations derived by Coller & Coller (1986) on the results of 96-well plates plated at 0.3 cells/well, it was calculated that the probability of monoclonality for the 6 hybrid cell lines on first cloning was between 0.90 and 0.95. The probability of monoclonality of hybridomas C4.6 and C4.19, which were cloned twice, was between 0.95 and 0.99. Apart from ensuring monoclonality, a reason for repeated cloning was to reduce the rate of chromosome loss. After 2 cycles of cloning, rate of chromosome loss is small but the risk of chromosome loss never ceases completely (Goding, 1986).

In summary, two MAbs (C4.6 and C4.19) have been successfully produced as tools for immunoblockade studies. Chapters 4, 7, 8 and 9 describe the further characterization of these and other MAbs and their subsequent application in pharmacological experiments.

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CHAPTER 4

Characterization, purification and fragmentation of monoclonal antibodies against CGRP

4.1. Introduction

The need for further characterization and purification of MAbs depends on their intended use. Since it was an objective of the project to understand the process of immunoblockade quantitatively, at least partial purification of MAbs was desirable. It was also necessary to select the MAb with the most desirable properties for use in pharmacological experiments. The isotype, affinity and specificity of 4 anti-CGRP MAbs were characterized in the present investigations. In addition, the utility of the MAbs for the immunocytochemical localization of CGRP was investigated.

A major objective of the project was to compare the application of whole immunoglobulins with antibody fragments in immunoblockade studies. The potential advantages of the use of antibody fragments have been discussed in Chapter 2. Immunoglobulins may be fragmented by controlled proteolysis. The portion of the polypeptide chain between the C_H1 and C_H2 domains of the heavy chain, known as the "hinge region", is susceptible to proteolysis. When an IgG molecule is subject to proteolytic attack by the enzyme papain in the presence of low concentrations of sulphydryl compounds, one or more peptide bonds in the hinge region are split, leading to the release of two identical Fab fragments (fragment having the antigen binding site) and a homogeneous Fc fragment (fragment that crystallizes).

The enzyme pepsin also cleaves at the hinge region but the cleavage site lies on the carboxyl side of the inter-heavy chain disulphide bonds. The resulting fragment is named $F(ab')_2$ because it contains two antigen binding sites. Fab' fragments may be produced from $F(ab')_2$ by reduction followed by alkylation. Mild conditions of reduction with cysteine are used to lyse the inter-heavy chain disulphide bonds whilst leaving the disulphide bond between the heavy and light chains intact. Fab' fragments are distinguished from Fab fragments which are univalent antigen binding fragments arising directly from digestion of IgG with papain. Fab' fragments are about 10% larger than Fab fragments but the small difference in molecular size is unlikely to be significant in immunoblockade studies.

4.2. Methods

4.2.1. ELISA, receptor binding assay and RIA

Materials	Supplier
HαCGRP, HβCGRP, RαCGRP, RβCGRP	Bachem or Peninsula
2-[^{125}I]-iodohistidyl^{10}-HαCGRP	Amersham
HαCGRP$_{8-37}$	Bachem
C-terminal 25-37 and Tyr^{0}-28-37 fragments of HαCGRP	Celltech
Substance P	Peninsula
Rat amylin	Peninsula

Four anti-CGRP MAbs (C4.6, C4.19, R1.50 and R2.73) from cell lines that had been cloned twice were characterized for their relative affinity for different forms of CGRP by the indirect ELISA described in Chapter 3. Dr. C. Plumpton (Clinical Pharmacology Unit, Addenbrooke's Hospital) provided the MAbs R1.50 and R2.73 which were raised against RαCGRP. The ability of the MAbs to inhibit CGRP receptor binding was compared in the rat liver membrane binding assay described in Chapter 5.

To allow modelling of immunoblockade in tissue bath experiments (Chapter 8), RIA was performed to estimate the dissociation constant (Kd) of MAb C4.19 and the maximum binding capacity (B_{max}) of the purified ascites. The RIA method is found in Chapter 3. The dilution of purified ascites that bound 50% of radiolabelled CGRP was used in the binding displacement experiments. The displacement of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP binding to MAb C4.19 by RαCGRP and RβCGRP was investigated.

The Kd and B_{max} of MAb C4.19 for RαCGRP and RβCGRP were estimated by fitting the following model to binding data from RIA displacement experiments:

$$B(C) = \frac{B_{\max} \times C}{Kd \times (1 + D/Kd_D) + C} + NS \times C$$

where B(C) is the binding B of ligand (2-[^{125}I]-iodohistidyl^{10}-HαCGRP) concentration C in the presence of displacer (RαCGRP or RβCGRP) concentration D. Kd and Kd_D are the dissociation constants of antibody binding to ligand and displacer respectively. B_{max} is the concentration of binding sites and NS is the non-specific binding parameter. Parameter estimation was performed using the non-linear extended least-squares regression package MKMODEL version 4.72 (Biosoft, Cambridge).

The CGRP binding site of MAb C4.19 and its potential cross-reactivity with relevant peptides were investigated by RIA and indirect ELISA. The ability of 1 μM HαCGRP$_{8-37}$ or substance P to displace 2-[^{125}I]-iodohistidyl^{10}-HαCGRP binding to MAb C4.19 was tested in the RIA. The potential of MAb C4.19 to bind C-terminal (8-37, 25-37 and Tyr^{0}-28-37) fragments of HαCGRP, substance P and rat amylin was tested by indirect ELISA. The same concentration of HαCGRP or other peptides (400 nM) was used to coat wells of the microtitre plate.

4.2.2. Determination of antibody class

Knowledge of antibody class and subclass is useful for determining the strategy for purification. For example, Protein A binds IgG with high affinity and specificity and Protein A affinity chromatography is a particularly relevant for IgG purification. Moreover, antibodies of different classes may exhibit different pharmacokinetic characteristics *in vivo* (Chapter 2).

MAbs were isotyped by the Ouchterlony immunodiffusion technique (Ouchterlony & Nilsson, 1986) according to the commercial kit supplier's instructions (Serotec). Ouchterlony analysis is an efficient method for the determination of antibody class because the technique is very sensitive and simple, is not antigen-dependent, and does not require enzyme- or radioisotope-labelled antisera. Tissue culture supernatant was used for Ouchterlony analysis because ascites fluid contains normal immunoglobulins which may lead to ambiguous results.

Approximately 75 μl of culture supernatant (from densely grown cloned hybridoma cells) was added to the large central well of the rosette. Ten μl of antisera directed against the 4 mouse IgG subclasses (IgG_1, IgG_{2a}, IgG_{2b}, IgG_3), IgA and IgM were added to each of the outer 6 wells of the same rosette. The plate was then tightly closed and diffusion allowed to proceed at room temperature for 24 to 48 hours until precipitation lines, which indicate the presence of a reaction, were visible.

4.2.3. Determination of protein concentration

Protein concentration was measured by a Coomassie brilliant blue dye binding assay (Bio-Rad) with bovine γ globulin as standard. The automated assay was performed in a 96-well microtitre plate. Ten μl of sample buffer (blank) was added to every well in column 1. Ten μl of protein standard (0.0625 mg/ml to 0.50 mg/ml) was added in triplicate in rows to wells of columns 2 to 4. Ten μl of appropriately diluted sample was added in duplicate to the remaining wells. Two hundred μl of Biorad dye reagent (diluted 1 in 5) was added to each well. The solutions were thoroughly mixed. Optical

density was measured at 600 nm after 5 to 10 minutes using a microtitre plate reader (Titertek Multiskan Plus Mk II, Flow Laboratories).

4.2.4. Purification of MAbs

4.2.4.1. Ammonium sulphate precipitation

4.2.4.1.1. Principles

Precipitation by salting-out to remove non-specific proteins is highly effective, simple and cheap. Concentration of purified protein may also be achieved by the procedure. Although other salts such as sodium sulphate may be used, the precipitation of antibodies is commonly performed by the use of ammonium sulphate.

Proteins in solution form hydrogen bonds with water through their exposed polar and ionic groups. When high concentrations of small, highly charged ions such as ammonium sulphate are added, these groups compete with proteins for binding to water. This removes water molecules from proteins and decreases their solubility, resulting in precipitation. The factors that will affect the concentration at which a particular protein will precipitate include the number and position of the polar groups, the molecular weight of the protein, the pH of the solution, and the temperature at which the precipitation is performed. The concentration (or percentage saturation) at which antibodies will precipitate varies from species to species. Fifty percent saturation is required for the precipitation of mouse antibodies. Most of the other components of serum and ascites fluid do not precipitate at this degree of saturation (Harlow & Lane, 1988).

Elevated levels of specific monoclonal immunoglobulins, as well as normal immunoglobulins and serum proteins, are present in ascites fluid. Ammonium sulphate precipitation is effective in removing serum proteins, particularly albumin, but normal immunoglobulins remain in ascites purified by this method. Some contamination with other high-molecular weight proteins will remain as well as proteins that are trapped in the large flocculant precipitates .

The required level of antibody purity is dependent on the application in mind, and ammonium sulphate precipitation may be readily combined with chromatographic methods if purer antibody preparations are needed.

4.2.4.1.2. Preparation of saturated ammonium sulphate solution

Saturated ammonium sulphate solution was prepared by adding 1 kg of ammonium sulphate crystals (BDH) to 1 litre of deionized water and stirring for 1 to 2 days at room temperature. Undissolved crystals was observed to be present in the solution.

The temperature of the solution was lowered to 4°C overnight and the pH adjusted to 7.4 with diluted ammonium hydroxide solution (BDH). The pH of small aliquots which had been diluted 1 in 20 was measured after each addition of ammonium hydroxide. The pH of the saturated solution was not measured directly because salt errors and junction potentials could produce errors of greater than 1 pH unit (Tijssen, 1985).

4.2.4.1.3. Experimental procedures: ascites fluid

Ascites fluid which was confirmed by ELISA to be active was pooled and centrifuged for 30 minutes at 48,000 *g* (20,000 rpm; Sorvall) at 4°C. The supernatant was recovered. Saturated ammonium sulphate was added dropwise to the ascites fluid with gentle stirring until a 50% saturated solution was achieved. A milky suspension was observed and this was stirred for 1 to 2 hours. The suspension was centrifuged for 30 minutes at 48,000 *g* (20,000 rpm; Sorvall) at 4°C. The supernatant was discarded. The pellet was resuspended in 50% saturated ammonium sulphate in PBS and the above spin repeated. The pellet was finally resuspended in the required volume of PBS (30 to 50% of the original ascites fluid volume). The solution was dialysed three times against 1000 volumes of PBS over 48 hours to remove residual ammonium sulphate. The dialysed solution was finally centrifuged for 30 minutes at 48,000 *g* (20,000 rpm; Sorvall) to remove denatured protein. Protein concentration was estimated by the Biorad assay described in Section 4.2.3. The purified ascites fluid was tested for activity by ELISA.

4.2.4.1.4. Experimental procedures: hybridoma culture supernatant

Forty ml of culture supernatant was mixed with 40 ml of saturated ammonium sulphate solution. The mixture stirred for 60 minutes and centrifuged at 48,000 *g* (20,000 rpm; Sorvall). The pellet was resuspended in 800 µl of PBS and the solution dialysed three times against 1000 volumes of PBS over 48 hours. The dialysate was centrifuged (10000 rpm; Hereaus) to remove denatured protein.

4.2.4.2. Protein A sepharose affinity chromatography

Equipment (all from Pharmacia)

Chromatography column (borosilicate glass; Pharmacia C10 column; 1 cm i.d.)
packed with Protein A sepharose CL-4B (Pharmacia).
Peristaltic pump P-1
Single path monitor UV1 (optical and control units)
Frac-100 fraction collector

Correction (page 95): Buffer formulas should read:-

Start Buffer pH 8.9

1.45 M glycine (chromatographically homogenous grade; BDH)
3 M sodium chloride
Adjusted to pH 8.9 with sodium hydroxide

Elution Buffer pH 3.0

100 mM citric acid
20 mM sodium hydroxide
Adjusted to pH 3.0 with sodium hydroxide

Chart recorder

Start Buffer pH 3.0

1.45 M glycine (chromatographically homogeneous grade; BDH)
20 mM sodium chloride
Adjusted to pH 3.0 with sodium hydroxide

Elution Buffer pH 8.9

100 mM citric acid
3 M sodium chloride
Adjusted to pH 8.9 with sodium hydroxide

Protein A-sepharose affinity chromatography was used to purify MAb R1.50 IgG and $F(ab')_2$ and Fab' fragments. Staphylococcal protein A binds to the Fc region of many IgG molecules and provides a method of IgG purification. It is also possible to separate purified IgG from Fab fragment which does not bind Protein A due to the lack of Fc region.

Two column volumes of start buffer (degassed) were run through the column followed by pre-cycling with elution buffer (degassed) until a stable chart recorder baseline was obtained. Start buffer was run through the column again. The sample was diluted with start buffer and loaded at 0.5 ml/min. Absorbance was monitored at 280 nm and 1 ml fractions collected. Following the first peak, IgG was eluted with elution buffer. At the end of the procedure, PBS/thiomersal 0.05% was run through the column for storage, and 70% ethanol was run through the pump and the detector flow cells.

4.2.5. Fragmentation of MAbs

4.2.5.1. Preparation of $F(ab')_2$ by pepsin digestion

Materials	**Supplier**
Pepsin	Boehringer Mannheim
Citric acid (anhydrous)	Sigma
Trisodium citrate	Sigma
MAb R1.50 IgG	C. Plumpton
MAb C4.19 IgG	K. Tan
Normal mouse IgG	Sigma

100 mM Citrate buffer pH 3.5

100mM citric acid was titrated against 100 mM trisodium citrate (100 mM citric acid: 19.21 g/l; 100 mM trisodium citrate: 29.41 g/l). Ratio is approximately 2:1 citric acid:trisodium citrate.

4.2.5.1.1. Experimental procedures

Pepsin is a non-specific protease which is only active at acid pH, and is irreversibly denatured at neutral or alkaline pH. Preliminary experiments were performed with Protein A affinity purified MAb R1.50 to determine optimum conditions. Two published methods were evaluated. The methods used the same protein:enzyme ratio but digestion was performed in 200 mM acetate buffer at pH 4.2 (Tijssen, 1985) or 100 mM citrate buffer at pH 3.5 (Parham, 1986). Forty-eight hour time course studies were performed. Samples were removed at 4, 8, 12, 24 and 48 hours from the incubation mixture and the reaction stopped. The extent of fragmentation was followed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The activity of the $F(ab')_2$ fragments produced was assessed by indirect ELISA.

The following optimized method was developed and was subsequently followed. IgG (or ammonium sulphate purified ascites fluid) was diluted to 5 mg/ml with 100 mM citrate buffer pH 3.5 (Antibodies in PBS could be diluted 1 in 5 without altering the pH of citrate buffer). Pepsin (1 mg/ml stock) was added to IgG in a ratio 1:100 (pepsin:IgG w/w) and the mixture incubated at 37°C for 4 hours. The reaction was stopped by addition of 1 M Tris HCl pH 9 to the mixture to give pH 7.4.

4.2.5.2. Preparation of Fab' fragments from $F(ab')_2$ fragments

Materials	**Supplier**
Anhydrous L-cysteine	Sigma
Iodoacetamide	Sigma
$F(ab')_2$ fragments prepared as above	

Reagents

1 M cysteine freshly prepared in 0.1 M Tris HCl pH7.4 (r.t.)/0.1 M NaCl

0.5 M iodoacetamide freshly prepared in 0.1 M Tris HCl pH7.4 (r.t.)/0.1 M NaCl (kept in dark).

4.2.5.2.1. Experimental procedures

Preliminary experiments investigated the effect of cysteine concentration on $F(ab')_2$ reduction and the following method was developed. The procedures were scaled up when necessary. Twenty µl 1 M cysteine was added to 2 ml pepsin digest or $F(ab')_2$ in

PBS (protein concentration 2 to 5 mg/ml), giving a final cysteine concentration of 10 mM. The mixture was incubated for 2 hours at 37°C. The reaction was stopped by addition of 300 µl 0.5 M iodoacetamide (alkylation step). After an incubation period of 1 hour at room temperature in the dark, the mixture was dialysed three times against 1000 volumes of phosphate buffered saline over 2 days. The activity of the anti-CGRP Fab' fragments produced was checked by indirect ELISA based on the avidin-biotin reaction (see Section 4.2.5.4.).

4.2.5.3. Concentration of F(ab')$_2$ and Fab' fragments

F(ab')$_2$ and Fab' fragments were concentrated by use of Centriprep-30 and Centriprep-10 concentrators (Amicon) with 30 kDa or 10 kDa molecular weight cutoffs respectively. Ultracentrifugation was performed according to the manufacturer's instructions. The protein concentration of the concentrated sample and the filtrate was determined. Protein was undetectable in the filtrate.

4.2.5.4. Indirect ELISA of F(ab')$_2$ and Fab' fragments

Materials	**Supplier**
Biotinylated goat anti-mouse (Fab specific) IgG	Sigma
Streptavidin-horseradish peroxidase	Vector Laboratories

The polyclonal rabbit anti-mouse reagent used in the routine ELISA consists of antibodies mainly directed against the Fc region. Therefore a modified ELISA which employed a biotinylated goat anti-mouse (Fab specific) IgG as the second antibody (1 in 1000) and streptavidin-horseradish peroxidase (1 in 500) was used for the detection of F(ab')$_2$ and Fab' fragments.

4.2.6. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Equipment

Mini-PROTEAN II slab cell (Biorad) consisting of lower buffer chamber, casting stand, inner cooling core and sandwich clamp assemblies.

Electrophoresis power supply EPS500/400 (Pharmacia)

Gilson pipettes fitted with Multiflex pipette tips (sample loading)

Biorad Gel Dryer model 543

Solutions

Acrylamide/Bis (30% total acrylamide; 2.67% cross-linker)

Acrylamide	29.2 g
N'N'-Bis-methylene-acrylamide (Bis)	0.8 g
Deionized water to	100 ml

Solution filtered with Whatman No.1 paper.

Sample buffer

Deionized water	20 ml
0.5 M Tris-HCl, pH 6.8	5 ml
Glycerol	4 ml
10% (w/v) SDS	8 ml
0.05% (w/v) Bromophenol blue	1 ml
2-β-mercaptoethanol	2 ml (or deionized water for non-reducing gel)

Electrode (running) buffer, pH 8.3

Tris base	3 g
Glycine	14.4 g
SDS	1 g
Deionized water to	1000 ml

Resolving (separating; lower) gel (10%)

Deionized water	4 ml
1.5M Tris-HCl, pH 8.8	2.5 ml
10% SDS	100 µl
Acrylamide/Bis solution	3.35 ml
10% ammonium persulphate (fresh)	50 µl
N,N,N',N'-tetramethylethylene-diamine (TEMED)	5 µl

Stacking (upper) gel

Deionized water	6.1 ml
0.5 M Tris-HCl, pH 6.8	2.5ml
10% SDS	100 µl
Acrylamide/Bis stock	1.3 ml
10% ammonium persulphate (fresh)	50 µl
TEMED	10 µl

Coomassie Blue staining solution

Coomassie Blue R-250	200 mg
Methanol	80 ml
Glacial acetic acid	20 ml
Deionized water to	200 ml

Destain solution

Methanol	400 ml
Glacial acetic acid	100 ml
Glycerol	10 ml
Deionized water to	1000 ml

Pre-stained molecular weight markers

The contents of vial (SDS-70B; Sigma) were dissolved in 0.4 ml twice-concentrated sample buffer. 0.4 ml 48% w/v urea was added and the mixture incubated at 37°C for 2 hours before aliquoting for storage at -20°C. Five µl was used for one lane of each gel. Pre-stained molecular weight markers from Biorad were also used in some experiments.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Laemmli buffer system (Laemmli, 1970) and the Biorad mini-PROTEAN slab cell which allowed analysis of protein samples in miniature gels. The acrylamide concentration used was 10% as this provided the best resolution for the analysis of antibodies and antibody fragments. The lower gel was poured, overlaid with water saturated isobutanol and allowed to polymerize for about 45 minutes. Liquid was removed and the upper gel poured with comb in place. The gel was allowed to polymerize over approximately 45 minutes and the comb removed. The wells were rinsed with deionized water followed by running buffer.

Samples were diluted with an equal volume of sample buffer and heated to 100°C for 2 to 5 minutes. Under such conditions proteins unfold and bind to SDS which imparts a very strong negative charge to the protein, dominating its native charge. The protein-SDS complexes possess essentially identical charge densities and electrophoretic mobility in acrylamide is inversely proportional to the logarithm of molecular weight. Five to 10 µg of protein was loaded into each well. Molecular weight markers were run in conjunction with the samples. The gel was run at 200 mV until the bromophenol blue line reached the bottom. The gel was placed into staining solution for at least 30 minutes, destained, and dried onto cellophane with a gel dryer (Biorad model 543).

The purity of immunoglobulins and Fab' fragments was determined by densitometric scanning of dried gels (Molecular Dynamics Personal Densitometer; ImageQuant software).

4.2.6.1. Analysis of antibody fragmentation by SDS-PAGE

SDS-PAGE allows the analysis of the cleavage of heavy chains, the cross-linking of polypeptide chains by disulphide bridges and the size of the fragments produced. SDS-PAGE can be performed under reducing or non-reducing conditions by preparing the samples in the presence or absence of the reducing agent β-mercaptoethanol. This permits the analysis disulphide bonds between IgG polypeptide chains.

Under non-reducing conditions, IgG migrates as a single protein band with molecular weight of approximately 150 kDa; under reducing conditions, IgG migrates as two bands corresponding to the heavy chain (~55 kDa) and the light chain (~25 kDa). $F(ab')_2$ under non-reducing condition migrates as a single band of ~ 110 kDa and when reduced is a doublet of bands at ~25 kDa. The lower band of the doublet corresponds to the light chain and the other is the NH_2-terminal half of the cleaved heavy chain (Fd'). Fab' migrates under non-reducing conditions as a single band of ~50 kDa and when reduced yields a doublet identical to that of $F(ab')_2$.

Fc under non-reducing and reducing conditions gives a single band of ~25kDa, migrating slower than the Fab doublet. This is not observed following pepsin digestion which breaks down the Fc fragment into smaller peptides (Goding, 1986).

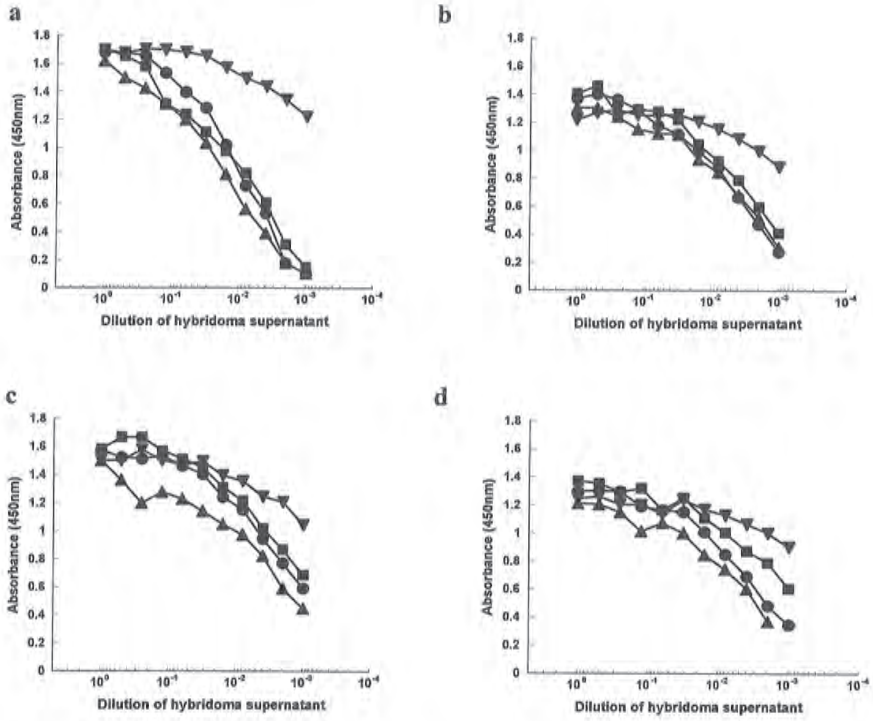

Figure 4.1: Binding of MAb C4.6 (triangles), C4.19 (circles), R1.50 (inverted triangles) and R2.73 (squares) to RαCGRP (a), RβCGRP (b), HαCGRP (c) and HβCGRP (d) by indirect ELISA. The same concentration of each form of CGRP was used to coat microtitre plate wells. Values are the mean of two observations.

4.2.7. Immunocytochemistry

The localization of CGRP-like containing structures in the rat spinal cord by MAbs C4.6, C4.19 and R1.50 was characterized by an immunoperoxidase method based on the avidin-biotin complex (ABC) system and by immunofluorescence. The principles and experimental procedures for immunostaining free-floating rat spinal cord sections using the ABC system are fully described in Chapter 6. Hybridoma supernatants were diluted 1 in 2 for immunostaining. Antibody specificity was tested by pre-absorption of MAbs with excess RαCGRP (2 μM) for 2 hours at room temperature.

Double immunofluorescence for the simultaneous localization of CGRP-like and substance P-like immunoreactivity was performed by the following procedures. Free floating rat spinal cord sections (30 μm) were blocked with 0.1 M PBS containing 3% normal horse serum, 0.1% BSA and 0.3% Triton-X 100 (blocking

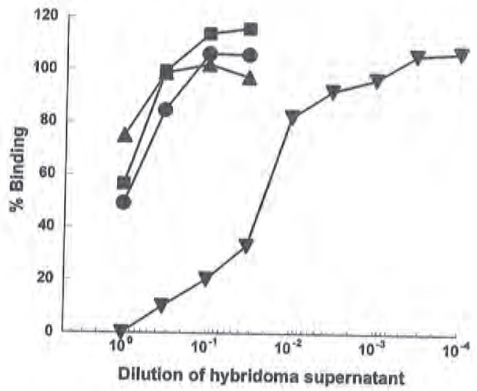

Figure 4.2: Effect of MAbs C4.6 (triangles), C4.19 (circles), R1.50 (inverted triangles) and R2.73 (squares) on the specific binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to rat liver membrane preparation. Binding was expressed as a percentage of total specific binding in the presence of buffer alone. Values are the mean of two observations.

reagent) followed by overnight incubation at 4°C with primary antibodies. The primary antibodies used were MAb C4.19 (hybridoma supernatant diluted 1 in 2 with blocking reagent) and a rabbit anti-substance P serum (Seralab; diluted 1 in 100 with blocking reagent). After washing, tissue sections were incubated for 2 hours with a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (Seralab; diluted 1 in 80) or a Texas Red-conjugated goat anti-mouse antibody (Biomeda; diluted 1 in 80). Tissue sections were washed, mounted on slides with an aqueous mountant (Vectashield), and viewed under a fluorescence microscope (see Chapter 6).

4.3. Results

4.3.1. ELISA, receptor binding assay and RIA

MAbs C4.6, C4.19, R1.50 and R2.73 cross-reacted with the α and β forms of human and rat CGRP by indirect ELISA (Figure 4.1). MAb R1.50 apparently had the highest relative affinity for all forms of CGRP but this was particularly evident for RαCGRP. MAb R1.50 was also the most effective MAb for inhibition of CGRP receptor binding (Figure 4.2).

The binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to MAb C4.19 was displaced by RαCGRP and RβCGRP in a concentration-dependent manner (Figure 4.3) but not by substance P or HαCGRP$_{8-37}$ at 1 μM. No difference in binding was

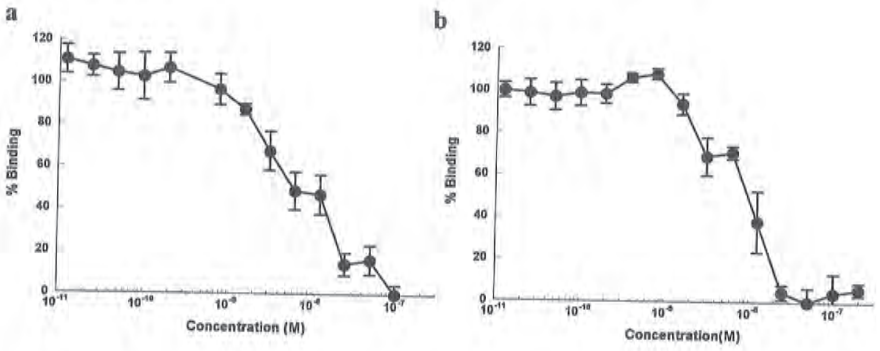

Figure 4.3: Displacement of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP binding to MAb C4.19 by RαCGRP (a) and RβCGRP (b). Binding was expressed as a percentage of total specific binding in the presence of buffer alone. Means and standard errors are plotted (n=4).

found whether assay buffer or Krebs buffer (Chapter 8) was used in the RIA. The dissociation constants (Kd) of MAb C4.19 for RαCGRP and RβCGRP were estimated to be 1.9 and 2.5 nM respectively. The estimated Kd and B_{max} (1.3 nmol/mg) were used for the simulation of *in vitro* immunoblockade (Chapter 8). MAb C4.19 did not bind to C-terminal (8-37, 25-37 and Tyr^0-28-37) fragments of HαCGRP, substance P and rat amylin by indirect ELISA (signal indistinguishable from background). The lack of cross-reactivity of MAb C4.19 with C-terminal fragments of CGRP by RIA and indirect ELISA suggests that it is directed against the N-terminus of CGRP.

4.3.2. Determination of antibody class

MAbs C4.6, C4.19, R1.50, and R2.73 were all found to be of the IgG_1 subclass.

4.3.3. Purification and fragmentation of MAbs

Cleavage of IgG to $F(ab')_2$ was clearly time dependent at pH 4.2, as shown by SDS-PAGE for MAb R1.50 (Figure 4.4). An incubation period of 48 hours was necessary for complete fragmentation at this pH. In contrast, fragmentation to $F(ab')_2$ was complete after 4 hours incubation at pH 3.5 (Figure 4.4) and this shorter procedure was chosen for further work.

The effective reduction of $F(ab')_2$ to Fab' fragments with 10 mM of cysteine was detected by the appearance of a band at approximately 45 kDa in the non-reduced SDS-PAGE gel. MAb R1.50 was successfully purified by Protein A affinity

chromatography and fragmented to $F(ab')_2$ and Fab' fragments (Figure 4.5). Large-scale fragmentation of MAb R1.50 led to Fab' fragments which were used in tissue bath experiments (Chapter 7).

MAb C4.19 was effectively purified by ammonium sulphate precipitation of ascites fluid (Figure 4.6). The purity of immunoglobulins in the antibody preparation purified by ammonium sulphate precipitation was 55%. Application of the experimental procedures originally optimized for MAb R1.50 to MAb C4.19 and normal mouse IgG also led to the successful production of Fab' fragments (Figures 4.6 and 4.7). The purity of the MAb C4.19 Fab' fragment produced was 92%. MAb C4.19 and normal mouse Fab' fragments were used for *in vivo* immunoblockade studies (Chapter 9).

Similar binding curves were observed when the same protein concentration of MAb R1.50 and MAb C4.19 IgG, $F(ab')_2$ and Fab' were used to detect RαCGRP by indirect ELISA (Figure 4.8).

4.3.4. Immunocytochemistry

CGRP-immunoreactive nerve fibres were detected by MAbs C4.6, C4.19 and R1.50 in the rat spinal cord (Figures 4.9 to 4.13). Specific immunostaining with the lowest background was observed with MAb C4.19. Immunostaining was abolished by pre-absorption with 2 μM CGRP (Figures 4.10). The highest density of CGRP-like immunoreactive nerve fibres was found in laminae I and II (Figure 4.11). Immunoreactive fibres were also detected around the central canal. Motoneurons in the ventral horn were characteristically immunostained (Figure 4.12). No difference in CGRP-like immunostaining was observed in the presence of 10 μM neurotensin or cholecystokinin. Double immunofluorescence showed that CGRP- and substance P-like immunoreactivity were co-localized in laminae I and II of the spinal cord (Figure 4.13).

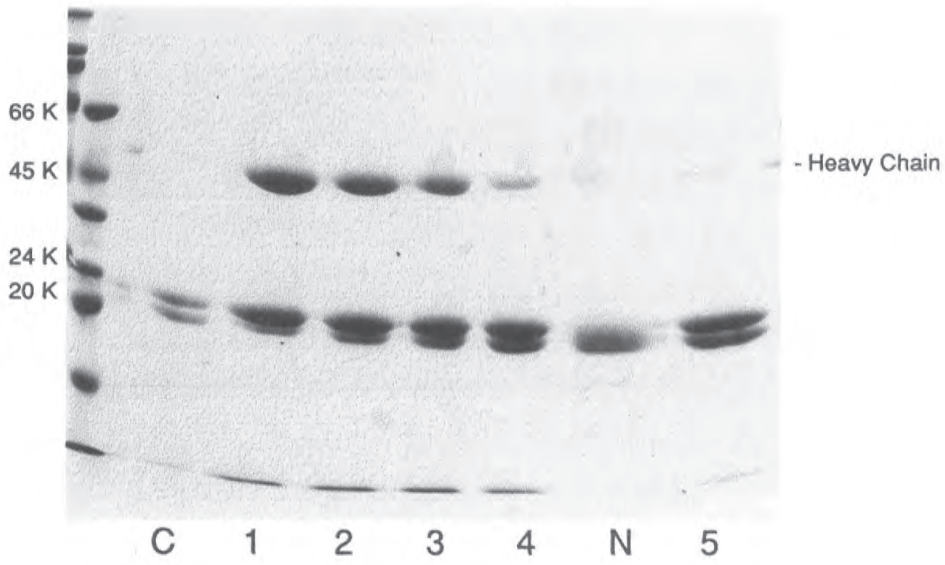

Figure 4.4: SDS-PAGE under reducing conditions of MAb R1.50 following incubation with pepsin in 200mM acetate buffer pH 4.2 for 4 (1), 8 (2), 12 (3), 24 (4) and 48 (5) hours. Like MAb R1.50, normal mouse IgG (N) was effectively fragmented, with disappearance of heavy chain, after 48 hours incubation with pepsin at pH 4.2. Complete fragmentation to $F(ab')_2$ was observed after incubation of MAb R1.50 IgG for only 4 hours with pepsin in 100mM citrate buffer at pH 3.5 (C). The concentration of acrylamide was 10%.

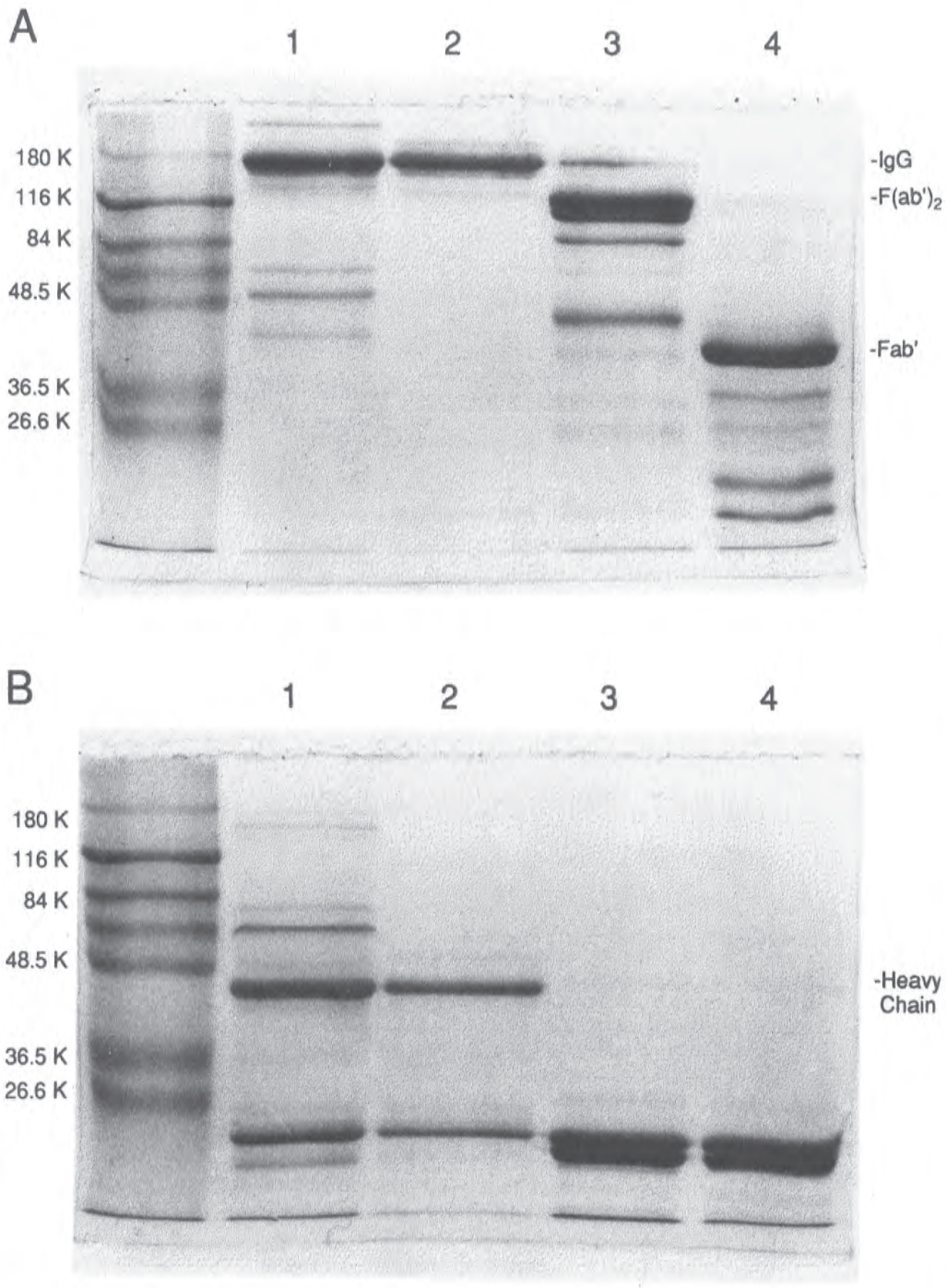

Figure 4.5: SDS-PAGE under non-reducing (A) and reducing (B) conditions of MAb R1.50 IgG purified from ascites fluid by ammonium sulphate precipitation (1), IgG purified by Protein A affinity chromatography (2), $F(ab')_2$ fragment (3) and Fab' fragment (4). The concentration of acrylamide was 10%.

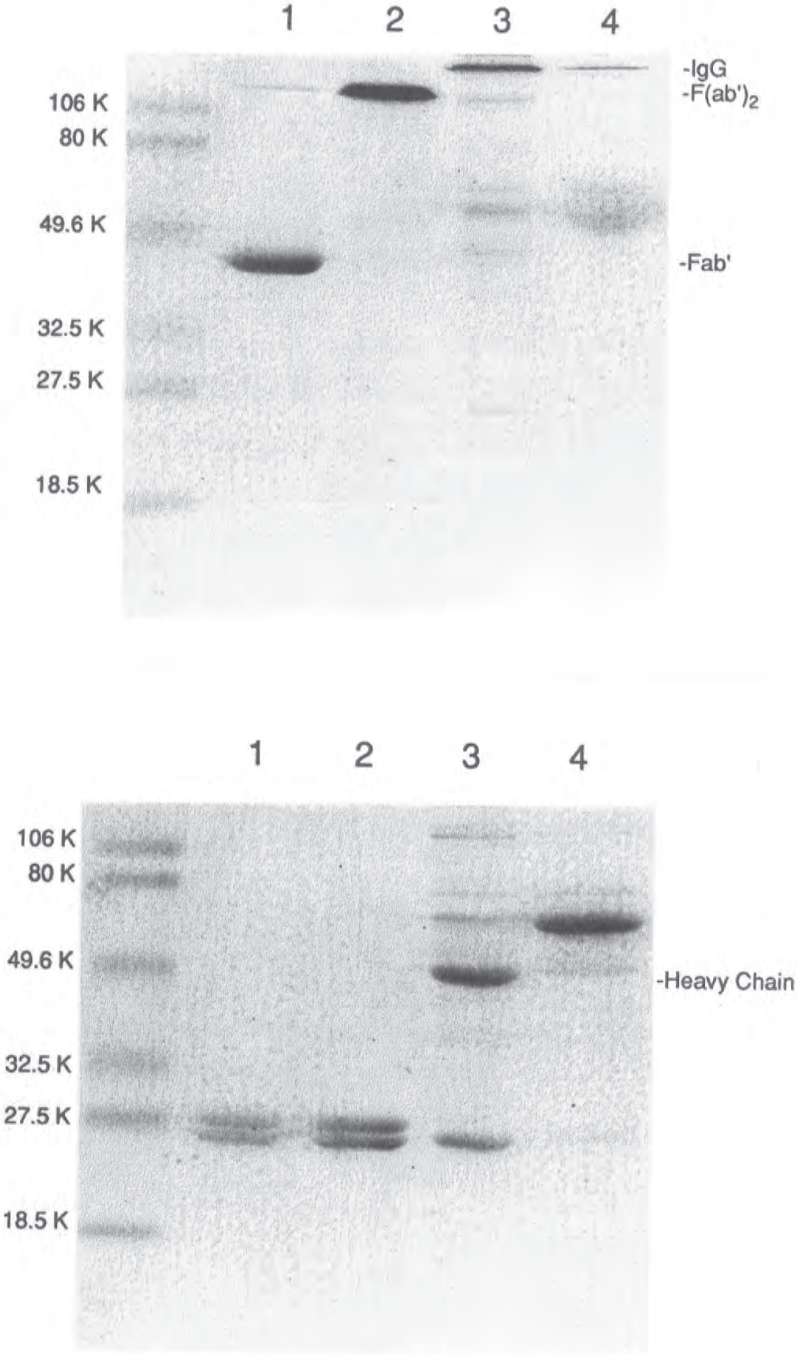

Figure 4.6: SDS-PAGE under non-reducing (top) and reducing (bottom) conditions of (1) MAb C4.19 Fab' fragment, (2) $F(ab')_2$ fragment, (3) IgG purified from ascites fluid by ammonium sulphate precipitation, and (4) neat ascites fluid. The concentration of acrylamide was 10%. The most abundant protein in ascites fluid was albumin (69kDa). The effectiveness of pepsin digestion is indicated by the complete disappearance of the heavy chain of IgG.

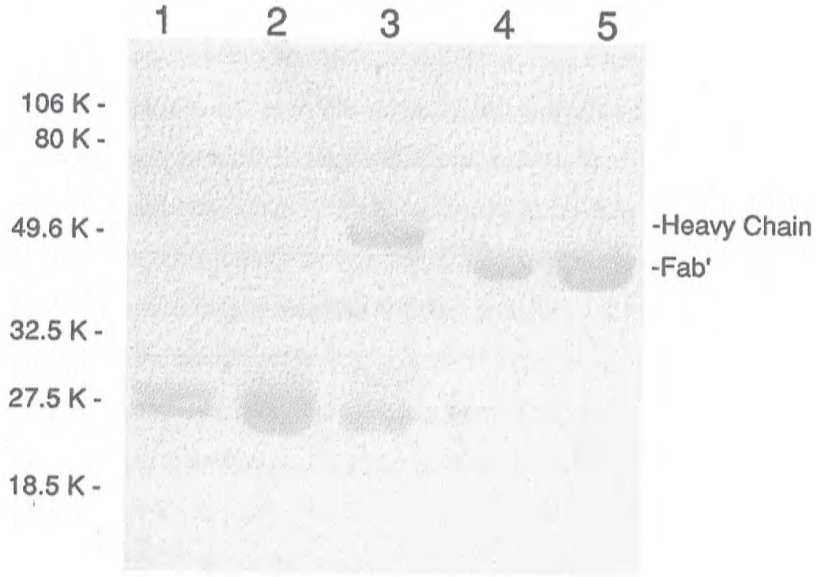

Figure 4.7: SDS-PAGE under reducing (1, 2, 3) and non-reducing (4, 5) conditions of normal mouse IgG (3), normal mouse Fab' fragment (2, 5) and MAb C4.19 Fab' fragment (1, 4). The concentration of acrylamide was 10%.

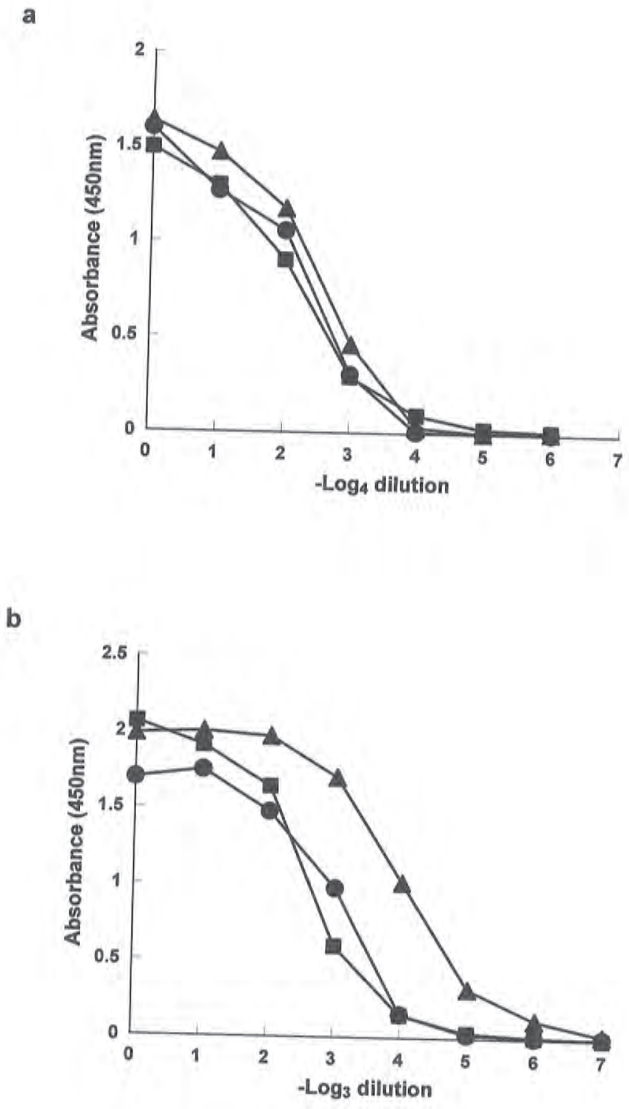

Figure 4.8: Binding of MAb R1.50 (a) and MAb C4.19 (b) IgG (squares), $F(ab')_2$ (triangles) and Fab' (circles) to RαCGRP by indirect ELISA. The starting protein concentration of MAb R1.50 and fragments was 20 μg/ml. The starting protein concentration of MAb C4.19 and fragments was 30 μg/ml. Values are the mean of two observations.

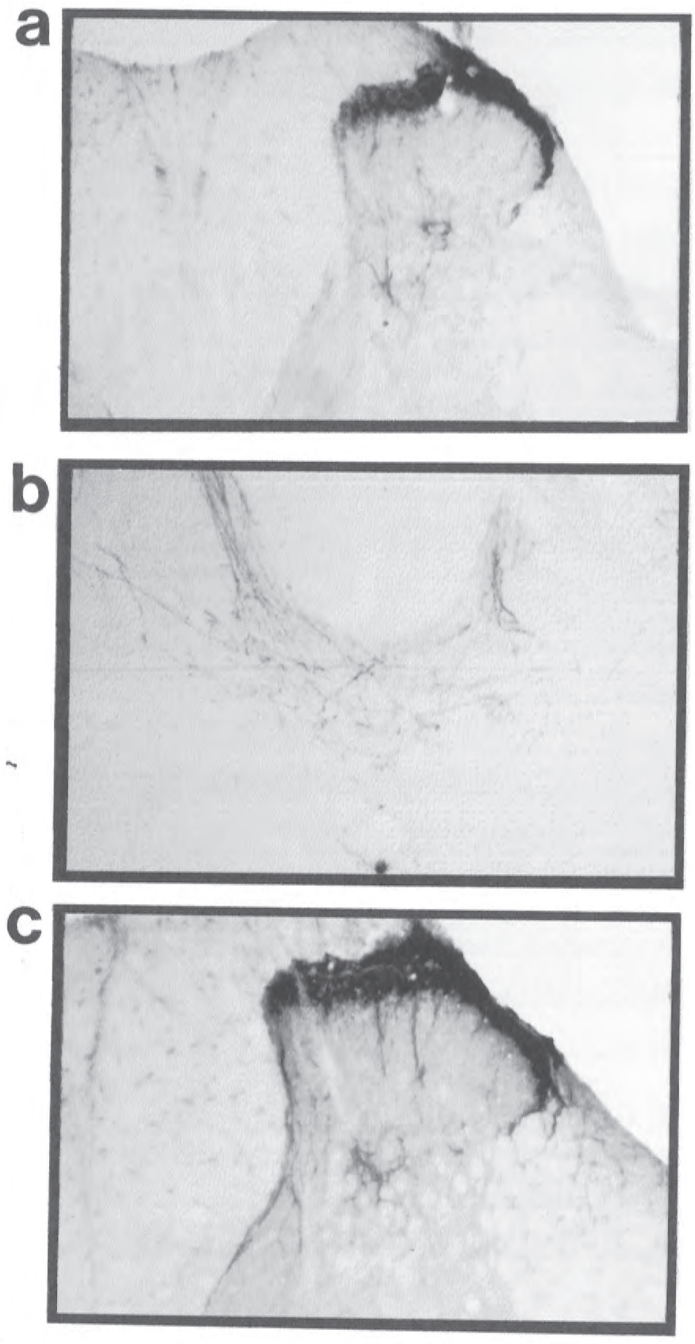

Figure 4.9: Immunostaining of nerve fibres in the dorsal horn and the area dorsal to the central canal of the rat spinal cord by MAb C4.6 (a,b x100). CGRP-immunoreactive fibres were also observed with MAb R1.50 (c; dorsal horn; x 100).

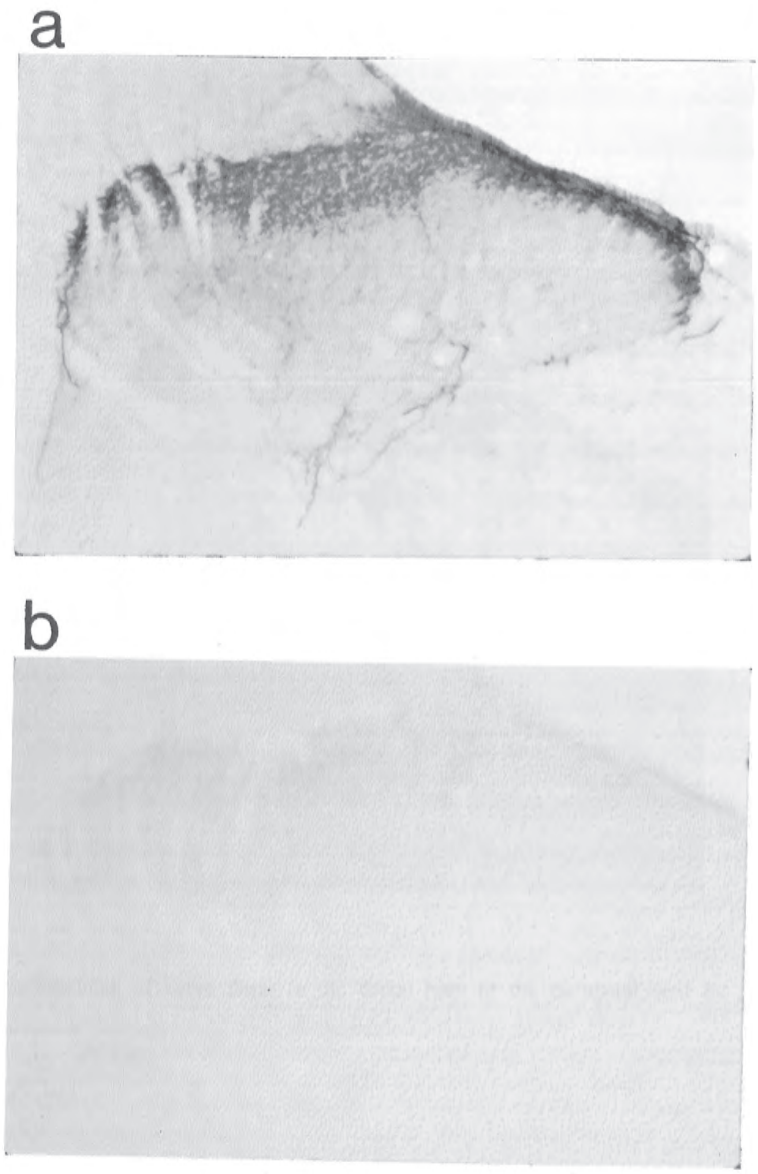

Figure 4.10: Demonstration of the specific immunostaining of nerve fibres by MAb C4.19 (a; x100) which could be abolished by pre-absorption with 2 μM RαCGRP for 2 hours at room temperature (b; x100). The dorsal horn of the rat spinal cord is shown.

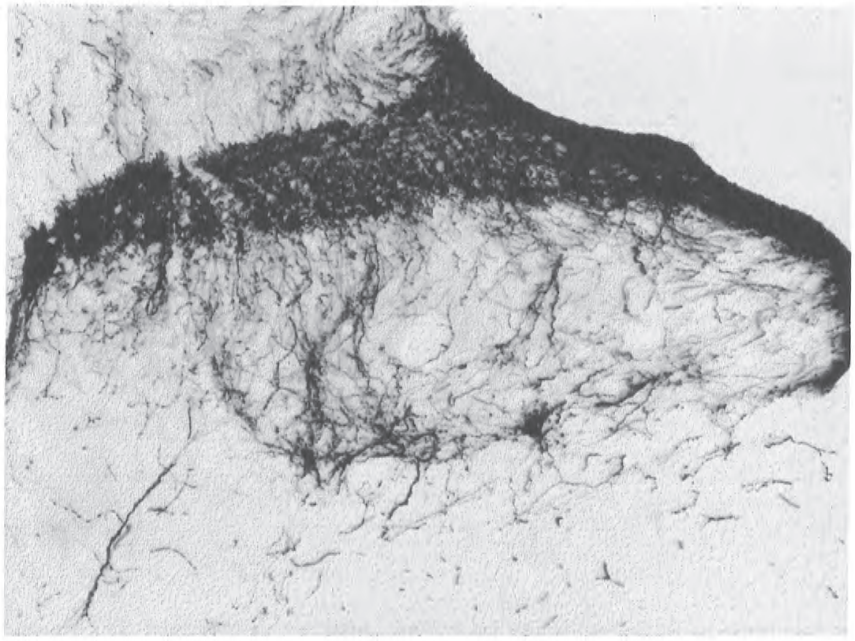

Figure 4.11: Immunostaining of nerve fibres in the dorsal horn of the rat spinal cord by MAb C4.19 (x100).

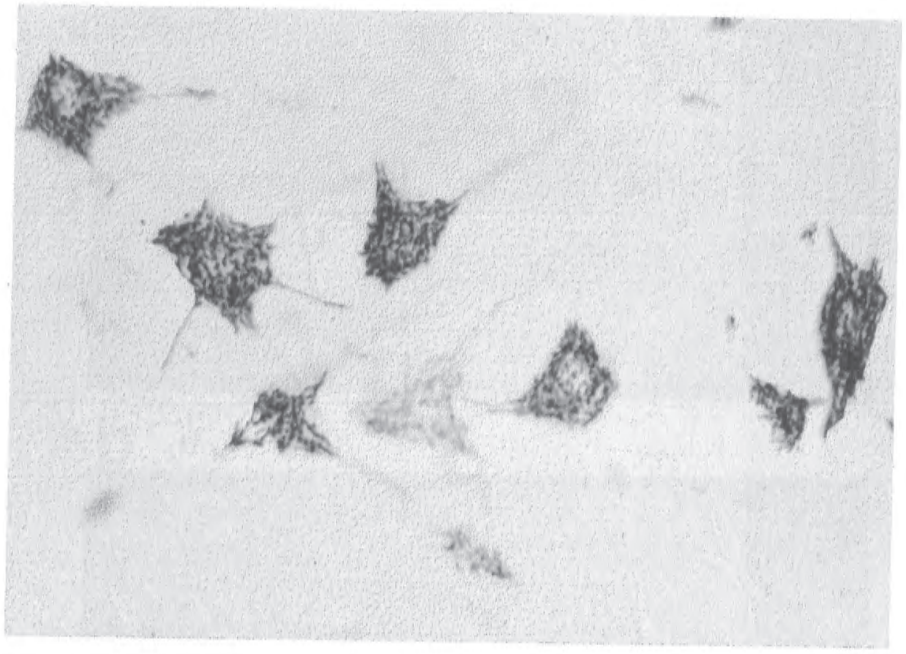

Figure 4.12: Immunostaining of motoneurons in the ventral horn of the rat spinal cord by MAb C4.19 (x100).

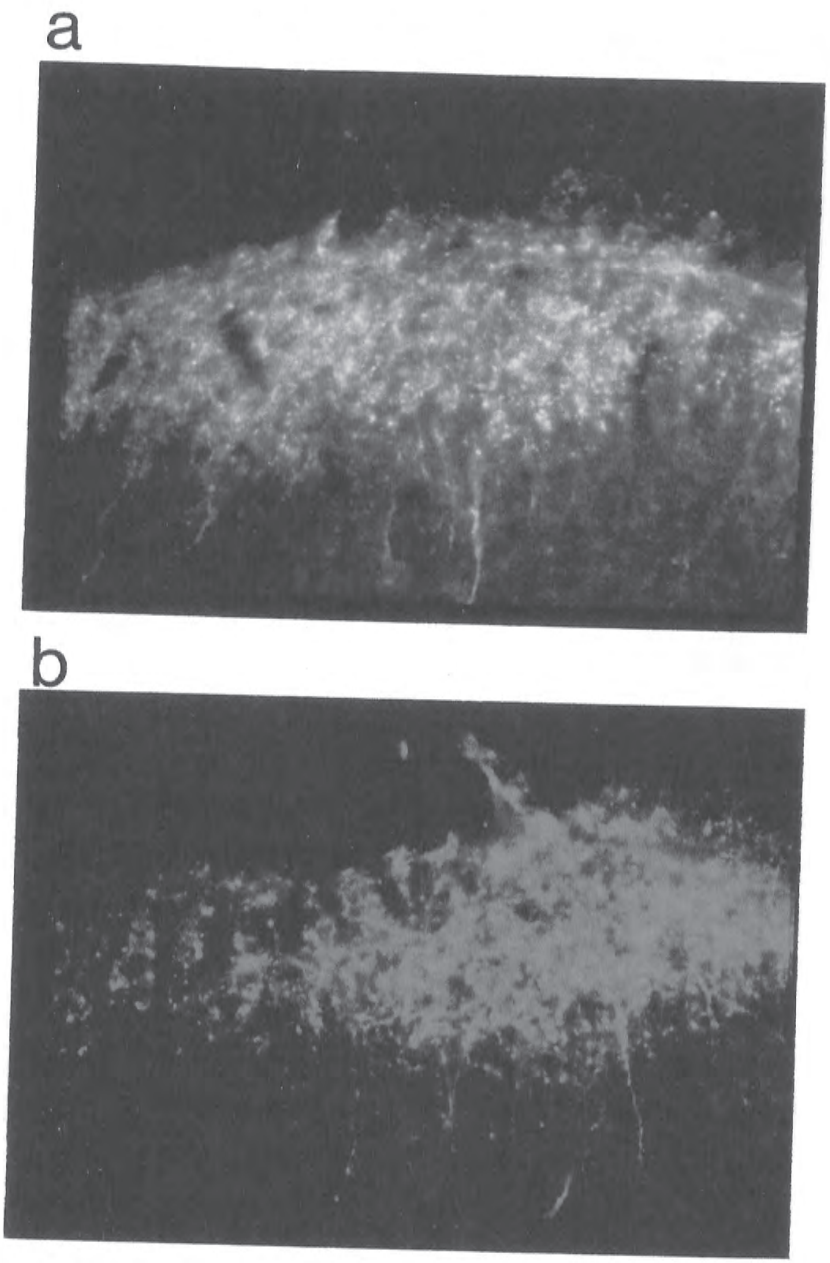

Figure 4.13: Double immunofluorescence staining of substance P- (a; FITC; x250) and CGRP- (b; Texas red; x250) immunoreactive nerves in the dorsal horn of the rat spinal cord by rabbit anti-substance P serum (Seralab) and MAb C4.19 respectively.

4.4 Discussion

Four MAbs have been purified and fragmented for pharmacological experiments. Biochemical characterization of the 4 candidate MAbs led to the initial choice of MAb R1.50 as the model MAb for immunoblockade studies (Chapter 7). However, all 4 MAbs were further assessed for their utility in immunoblockade experiments (Chapter 8), and MAb C4.19 was eventually used to investigate the physiological role of CGRP (Chapters 8 and 9).

Ammonium sulphate precipitation was particularly useful as a purification method because it also facilitated the concentration of MAbs. Concentrated MAb solutions allowed the addition of small volumes to tissue baths and injection of low volumes *in vivo*. In general, ascites fluid purified by ammonium sulphate precipitation was considered sufficiently pure for pharmacological experiments. Whilst purer antibody preparations may offer advantages, each purification step also potentiates the risk of denaturing antibodies. The concentration of specific antibody in purified ascites fluid may be obtained by estimating B_{max} from RIA experiments, as performed for MAb C4.19.

Protein A affinity chromatography is considered to produce low yields for mouse IgG_1 (Goding, 1986). However, this problem was overcome by the use of high salt concentrations and high pH which improve the binding of IgG_1 to protein A. Protein A affinity chromatography yielded highly pure MAb R1.50 and offered a method for the separation of IgG and $F(ab')_2$ fragments. However, the fragmentation of pure IgG to $F(ab')_2$ under optimized conditions was clearly complete and purification of the pepsin digestion mixture proved unnecessary. Further experience showed that pepsin treatment of ascites fluid purified by ammonium sulphate precipitation yielded pure $F(ab')_2$ as assessed by SDS-PAGE. These observations are in agreement with those of Parham (1983) who reported that pepsin treatment of ascites fluid at pH 3.5 degraded almost all proteins except IgG_1 to small molecular weight peptides. Thus, contrary to the recommendation by Lamoyi (1986), complete purification of IgG prior to pepsin digestion was unnecessary.

The different IgG subclasses vary in their susceptibility to proteolytic cleavage (Parham, 1983). IgG_1 is more resistant to papain than other IgG subclasses (Goding, 1986), and all attempts to produce MAb R1.50 Fab fragments by papain digestion failed. IgG_1 is also resistant to pepsin but the appropriate optimization of time and pH parameters led to excellent yields of MAb R1.50 $F(ab')_2$ fragments. Fab' fragments were successfully produced by reduction of $F(ab')_2$ fragments.

Optimization of the fragmentation procedure for each MAb may be necessary because individual MAbs appear to be unique in their susceptibility to fragmentation

(Parham, 1986). Indeed, pepsin digestion has been reported to rapidly destroy some MAbs (Lamoyi, 1986). However, application of the procedures optimized for MAb R1.50 to MAb C4.19 and normal mouse IgG, initially on an analytical scale, led to equally successful fragmentation. This could be related to the fact that MAb C4.19 is also an IgG_1 and that IgG_1 is a major IgG subclass in normal mouse serum. MAb R1.50 and MAb C4.19 fragments retained binding activity. The ELISA results usefully indicated the relative binding activity of IgG and the fragments at the same protein concentration. The greater binding activity of MAb C4.19 F(ab')2 fragments at a lower molar concentration than Fab' fragments is probably due to the higher avidity of bivalent molecules.

Specific immunostaining with very low background was found when MAb C4.19 was used for immunocytochemistry. The spinal cord was used as a model tissue for the assessment of the utility of MAbs in immunocytochemistry because patterns of CGRP immunoreactivity has been well characterized in this tissue with CGRP antisera (Gibson *et al.*, 1984). Both αCGRP and βCGRP are found in the rat spinal cord. Although the concentration of αCGRP may be 3 to 6 times higher than βCGRP in the dorsal spinal cord (Mulderry *et al.*, 1988), the CGRP immunoreactivity detected by MAb C4.19 probably relate to both forms of CGRP.

The role of CGRP in the dorsal horn of the spinal cord may include potentiation of synaptic transmission by altering calcium conductance (Ryu *et al.*, 1988; Oku *et al.*, 1988) and by increasing the release of substance P (Oku *et al.*, 1987). The presence of CGRP in motoneurons and its co-localization with choline acetyl transferase has led to suggestions that CGRP may be involved in neuronal regeneration, astroglial gene expression and enhancement of cholinergic transmission at the neuromuscular junction (Takami *et al.*, 1985; Streit *et al.*, 1989; Haas *et al.*, 1991; New & Mudge, 1986; Jinnai *et al.*, 1989).

Double immunofluorescence was shown to be possible using MAb C4.19 and an anti-substance P rabbit antiserum. Double immunostaining with MAb C4.19 is being used to examine physiologically important issues in collaboration with others.

In summary, 4 MAbs against CGRP have been purified, concentrated, fragmented and characterized with immunoblockade experiments in mind. MAb R1.50 emerged as a promising candidate MAb for pharmacological studies but all 4 MAbs were subsequently screened pharmacologically. MAb C4.19 also proved to be a useful tool for immunocytochemistry.

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CHAPTER 5

Development of monoclonal antibodies against the CGRP receptor

5.1. Introduction

A number of approaches to the development of MAbs against receptors have been advocated (reviewed in Chapter 1). The most direct route to anti-receptor antibodies is to immunize with the receptor itself. In practice, animals have been immunized with receptor-rich crude membrane preparations, whole cells which express the relevant receptor, affinity purified receptor preparation, peptide fragments of sequenced receptors or receptor protein bands identified in SDS-PAGE.

Methods which do not require purification of the CGRP receptor were investigated in this project since receptor purification is time-consuming and no published methods were available when the studies were started. This chapter details attempts to develop anti-CGRP receptor MAbs by *in vivo* and *in vitro* immunization with membrane preparations. *In vitro* immunization in culture (Reading, 1982, 1986; Boss, 1984, 1986) was investigated because it offered a number of advantages over conventional *in vivo* immunization, particularly when crude membranes were used as immunogens. An auto-anti-idiotypic approach was also pursued in collaboration. The characterization of MAbs developed with the auto-anti-idiotypic approach is described in Chapter 6.

5.2. Methods

Materials	**Supplier**
RαCGRP	Peninsula
Ethylene-diamine-tetraacetic acid (EDTA)	Sigma
Tris(hydroxymethyl)aminomethane (Tris)	Sigma
Phenylmethyl-sulphonyl-fluoride (PMSF)	Sigma
Bovine serum albumin (BSA)	Sigma
Bacitracin	Sigma
Aprotinin	Bayer
Magnesium Chloride	Amersham
Polyethyleneimine	Sigma

5.2.1. Preparation of rat liver membranes

Buffers

Buffer A:	0.1 mM PMSF in 50 mM Tris HCl pH 7.4
Buffer B:	0.5 mM EDTA
	0.1 mM PMSF in 50 mM Tris HCl pH 7.4

Wistar rats were killed by stunning and cervical dislocation. Livers were dissected out, immediately placed on ice, and transferred to a liquid nitrogen container as soon as possible for storage. Livers were thawed and their wet weight determined. All procedures during membrane preparation were performed at 4°C as far as possible. Buffer A was added at a volume (ml) equivalent to five times the wet weight (g). The livers were chopped up with scissors and tissue homogenized in short bursts using a Polytron homogenizer (Kinematica, Switzerland). The homogenate was centrifuged at 15000 *g* for 20 minutes at 4°C in a RC5C Sorvall Instruments (Dupont) centrifuge. Supernatant was decanted and centrifuged at 48000 *g* for 60 minutes at 4°C. The pellet was resuspended in Buffer B at a volume (ml) equivalent to five times the wet weight (g) and the suspension centrifuged at 48000 *g* for 60 minutes at 4°C. The pellet was finally resuspended in Buffer A. The membrane preparation was aliquoted and stored at -70°C. Protein concentration was determined as described below.

5.2.2. Preparation of bovine cerebellum membranes

Bovine cerebellum was collected from the local abattoir, transported to the laboratory in ice, and membranes prepared as described above for the rat liver.

5.2.3. Determination of protein concentration of membrane preparations

Protein concentration was measured by the Coomassie brilliant blue dye binding assay (Bio-Rad) described in Chapter 4. Bovine γ-globulin was used as the protein standard.

5.2.4. Receptor binding assay of CGRP using rat liver membrane preparation

Binding buffer

Aprotinin	20 u/ml
Bacitracin	0.1%
PMSF	0.1 mM

Magnesium chloride	5 mM
BSA	0.5%
Tris HCl pH 7.4 (at 4°C)	50 mM

Wash buffer

BSA	0.5%
Tris HCl pH 7.4 (at 4°C)	50 mM

The incubation mixture consisted of 50 µl binding buffer, 50 µl RαCGRP (10^{-12} to 10^{-6} M) or binding buffer (total bindable counts), 50 µl 2-[^{125}I]-iodohistidyl^{10}-HαCGRP (40,000 counts per minute [cpm]) and 100 µl membrane suspension (3 mg/ml). The tubes were gently shaken and then incubated at 4°C for two hours. The incubation mixture was vacuum filtered through GF/C filter paper (Whatman) that had been pre-soaked in polyethyleneimine (250 µl in 200 ml 50 mM Tris HCl pH 7.4). The filter paper was washed three times with 3 ml ice-cold wash buffer. Individual pieces of filter paper were placed in polypropylene tubes and radioactivity counted for 3 minutes in a Beckman Gamma 5500 counter.

5.2.4.1. Reduction of non-specific binding by siliconization and use of BSA

An experiment was designed to investigate the non-specific binding of CGRP. The effect of co-incubation with BSA and the siliconization of glassware and polypropylene materials on non-specific binding was tested. Glass and polypropylene tubes were siliconized by rinsing with Sigmacote® (Sigma) and drying in air. One ml aliquots of a 2-[^{125}I]-iodohistidyl^{10}-HαCGRP solution containing 50,000 cpm with or without 0.5% BSA were added to glass or polypropylene tubes. Samples (80 µl) were taken at timed intervals over 1 hour for radioactivity counting.

5.2.4.2. Estimation of receptor binding parameters

One or two-site binding models were fitted to the binding displacement data by non-linear least squares regression (MKMODEL version 4.72; Biosoft, Cambridge) with weighting factor inversely proportional to the square of the predicted bound concentration. The structural model parameters estimated were Kd (dissociation constant), B_{max} (concentration of binding sites), and the non-specific binding constant (NS). Initial Kd and B_{max} values were estimated by the method of DeBlasi *et al.* (1989). The Kd, B_{max} and NS of the displacer (cold ligand) were fixed at the values of the parameters of the radioactive-labelled ligand.

5.2.5. Use of receptor binding assay for screening serum and cell culture supernatants

The incubation mixture consisted of 50 µl serum or supernatant, 50 µl binding buffer, 50 µl 2-[^{125}I]-iodohistidyl^{10}-HαCGRP (40,000 cpm) and 100 µl membrane suspension (3 mg/ml). Non-specific binding was assessed by addition of excess RαCGRP (10^{-6}M) to the incubation mixture. Cell culture medium, myeloma supernatant and normal mouse immunoglobulin (or serum) were added to control tubes. The mixture was incubated overnight at 4°C before filtration.

An alternative procedure was used to screen supernatants obtained by the *in vitro* immunization protocol. Supernatants and membrane preparation were co-incubated for 5 days at 4°C prior to addition of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP and further incubation for 2 hours at 4°C.

5.2.6. Receptor binding assay of CGRP using bovine cerebellum membrane preparation

Specific binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to bovine cerebellum membrane was confirmed using the binding assay developed for rat liver membrane preparation.

5.2.7. Dot immunobinding assay for immunoglobulin in supernatants

Reagents

Phosphate buffered saline pH 7.4 (PBS; formula in Chapter 3)

Tween 20 (Sigma; 0.05% v/v)

4-chloro-1-naphthol (Sigma; 3 mg/ml freshly made in methanol)

Horseradish peroxidase conjugated rabbit anti-mouse antibody (ICN Flow; 1 in 1000)

Substrate reagent

Deionized water	20 ml (5 parts)
4-chloro-1-naphthol	4 ml (1 part)
Hydrogen peroxide 6%	40 µl (0.01% final concentration)

A dot immunobinding assay was used to screen for immunoglobulin secretion by hybridomas. The following procedures were adapted from those described by Hawkes (1986). A sheet of 0.45 µm nitrocellulose membrane filter paper (Schleicher and Schuell) was lined with pencil and ruler to outline square areas. Half to one µl of

supernatant from fusion was added to the centre of each square and the blots allowed to dry. Non-immune mouse immunoglobulin (10 µg/ml; Sigma) was used as a positive control. Myeloma culture supernatant and fresh cell culture medium were used as negative controls. The filter paper was washed three times with PBS/Tween 20 0.05%. The filter paper was left to soak for several minutes between each wash. PBS/Tween 20 0.05% was removed and approximately 30 ml of PBS/BSA 1% was added (blocking step). The filter paper was left to soak in this solution for 15 to 30 minutes. PBS/BSA 1% was removed and 30 ml horseradish peroxidase conjugated rabbit anti-mouse antibody (1 in 1000 in PBS/Tween 20 0.05%/BSA 1%) added and left for 1 hour. After washing the filter paper three times with PBS/Tween 20 0.05%, substrate reagent was added and left to soak until dots appeared. The filter paper was rinsed with deionized water, dried and photocopied if required.

5.2.8. *In vivo* immunization protocol and screening for anti-receptor antibodies

Four 5 to 6 week old female Balb/C mice were immunized with rat liver membrane preparation on 4 occasions at monthly intervals with 20, 4, 4 and 14 mg of protein in 200 µl respectively. Another group of 14 Balb/C mice were immunized with 0.7 to 1 mg of rat liver membrane preparation on 5 occasions. Ten Balb/C mice and four 10-week-old female LOU/C rats were immunized with 1 mg of bovine cerebellum membrane preparation 3 times at monthly intervals. The i.p. route was used for all injections. Blood was collected from mice by tail bleed 7 days after booster injections and serum tested in the receptor binding assay.

5.2.9. *In vitro* immunization protocol and fusion

Culture medium

		Stock solution	*Supplier*
50 µM 2-mercaptoethanol	0.5 ml	50 mM	Gibco
20mM HEPES	10 ml	1 M	Northumbria
1mM sodium pyruvate	5 ml	100 mM	Gibco
2% non-essential amino acids	10 ml	100 x	Flow
Foetal calf serum (20%)	100 ml		GlobePharm
Dulbecco's modified Eagles medium*	500 ml		Flow

*containing L-glutamine, penicillin 500 iu/ml, streptomycin 50 µg/ml, Amphotericin B 2.5 µg/ml.

Sterile stock solutions of the above materials were obtained from manufacturers.

However, adjuvant peptide (N-acetylmuramyl-L-alanyl-D-isoglutamine; Sigma) was not supplied sterile. Five mg was stored at -20°C, thawed, and made up in 5 ml fresh SFD under sterile conditions. This was dispensed as twelve 400 µl aliquots into cryovials and stored at -70°C. The remaining 200 µl was left in a Bijou tube, sealed and left in an incubator but no growth was detected. The rat liver membrane preparation was washed twice in PBS by centrifuging at 48,000 *g* for 1 hour and sterilized by γ-irradiation at 2.5 mRads (Department of Radiotherapy, Addenbrooke's Hospital).

One mg of rat liver membrane preparation was added to 10 ml culture medium in a 75 cm^2 flask. Ten ml culture medium was warmed to 37°C in a water bath. The spleen was removed from a Balb/C mouse into ice cold SFD and washed once in 10 ml SFD. Spleen cells were suspended in 10 ml SFD and centrifuged for 5 minutes at 1500 rpm (Denley). The pellet was resuspended in 10 ml SFD and centrifugation repeated. The pellet was then resuspended in 10 ml warm (37°C) medium (prepared as above) and transferred to the 75 cm^2 flask. An aliquot of adjuvant peptide (400 µl) was added to the flask. The flask was kept with a loose lid in a humidified incubator (IR1500 5% CO_2, 37°C; Flow).

Blast cells were visible under the microscope after two days. Fusion with myeloma cells was performed as described in Chapter 3 four days after immunization.

The strategy was to screen initially for hybridomas producing immunoglobulins using the dot immunobinding assay, expand positive lines in 24-well plates so that cells could be cryopreserved and more supernatants obtained for screening and, finally, to screen supernatants with the receptor binding assay.

5.3. Results

5.3.1. Receptor binding assay

The binding assay was developed by adapting assay methods reported in the literature (Nakamuta *et al.*, 1986) and binding assays for other peptides used in the Unit. Non-specific binding, determined by addition of excess of unlabelled RαCGRP (1µM), was of the order of 20%. The importance of co-incubation with BSA and the siliconization of both glassware and polypropylene material in reducing non-specific binding was clearly demonstrated (Figure 5.1). The combined use of siliconization and BSA was particularly effective for polypropylene material. These findings clearly have implications for experiments other than the binding assay. Efforts were made to use siliconized glass and polypropylene materials and to dissolve CGRP in BSA-containing solutions whenever possible.

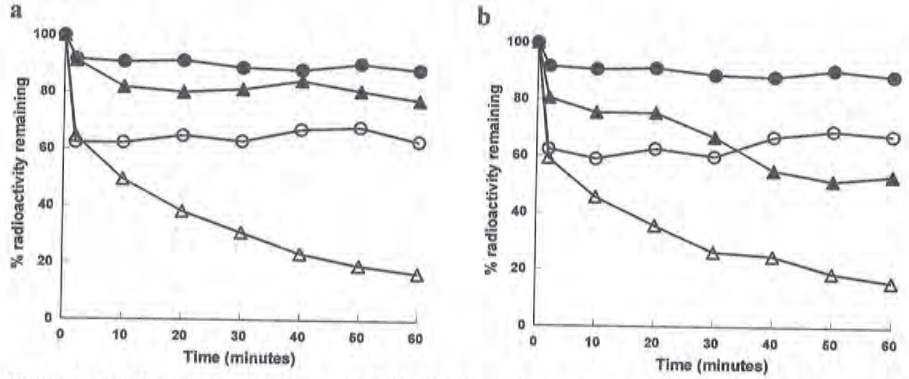

Figure 5.1: Effect of siliconization and BSA (0.5%) co-incubation on the non-specific binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to glass (a) and polypropylene (b) tubes over time. Non-specific binding is indicated by the loss of radioactivity in solution (single observations). Values from non-siliconized and siliconized materials (without BSA) are shown in open triangles and open circles respectively. The effect of BSA co-incubation on non-specific binding to non-siliconized and siliconized tubes is shown with closed triangles and closed circles respectively.

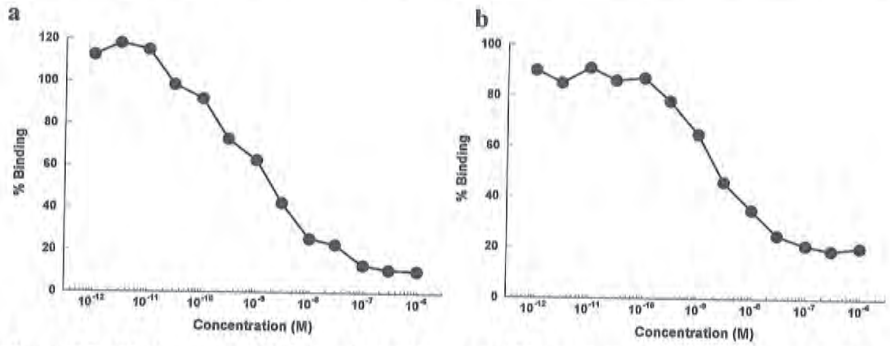

Figure 5.2: Displacement of the specific binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to rat liver (a) and bovine cerebellum (b) membrane preparations. Values are the mean of two observations.

Specific binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP was determined in rat liver and bovine cerebellum membrane preparations used for immunization (Figure 5.2). High concentrations of atrial natriuretic factor (0.1 μM), neuropeptide Y (2 μM), substance P (10 μM) and neurokinin A (2 μM) did not displace the specific binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP in the assay. Cell culture medium containing HAT, penicillin, streptomycin, amphotericin and HEPES-azide did not inhibit binding, thus permitting the use of the assay in screening hybridoma supernatants.

Table 5.1: Coefficient of variation of binding assay results

Addition to incubation mixture (n=4)	Coefficient of variation (%)
Buffer (total binding)	3.0
Culture medium (20FD/HAT/Azide)	1.2
Normal mouse immunoglobulin (10 μg/ml)	2.1
RαCGRP (1 μM; non-specific binding)	6.5

Coefficient of variation was calculated by division of the standard deviation by the mean and multiplication by 100.

The estimated Kd and B_{max} were 1.7 nM and 400 fmol/mg protein respectively for the binding of CGRP to the rat liver membrane receptor. The one-site model fitted the data better than the two-site model as assessed by examination of residuals, coefficient of the variation of the parameters and the Schwartz information criterion.

Within the same assay, the coefficient of variation (standard deviation divided by mean) was evaluated for total binding and binding in the presence of culture medium, normal mouse immunoglobulin and excess RαCGRP (non-specific binding) using four replicates. The results are summarized in Table 5.1. The low coefficients of variation suggest that the assay is highly reproducible, at least within run.

5.3.2. *In vivo* immunization

Serum from mice immunized up to 5 times with different quantities of rat liver membrane did not inhibit the binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP in the binding assay. Figure 5.3 shows the results of one of the serum screening assays for anti-CGRP receptor antibodies. Similarly serum from rats and mice immunized with bovine cerebellum membrane were negative in the binding assay. Therefore, no fusions were attempted with spleens from these animals.

5.3.3. *In vitro* immunization

High fusion frequency was achieved with 100% of wells in 96-well plates producing hybridoma clones. All 186 supernatants screened in the dot immunobinding assay were positive for immunoglobulin secretion. The intensity of the dots were comparable to that due to normal mouse immunoglobulin at 10 μg/ml. Thus 186 cell lines were expanded in 24-well plates and cryopreserved. The hybridomas were allowed to overgrow prior to testing of the supernatants. Eighteen of the 186 supernatants tested in the binding assay apparently inhibited binding of 2-[^{125}I]-

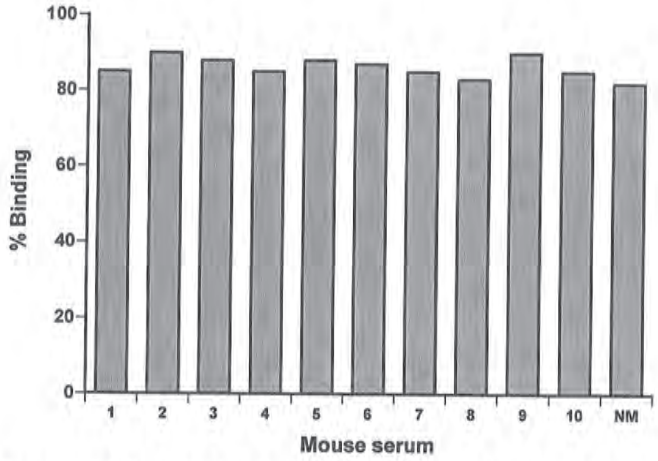

Figure 5.3: Effect of serum from mice (1 to 10) immunized 5 times with 0.7 to 1 mg of rat liver membrane preparation on the specific binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to rat liver membranes. Sera were collected 7 days after the last immunization and diluted 1 in 5 in the binding assay. Non-immune mouse serum (NM) was used as a control. Values are the mean of two observations.

iodohistidyl^{10}-HαCGRP by 30% or greater compared with normal mouse immunoglobulin. The hybridomas were therefore thawed, grown to confluence and their supernatants re-tested in the binding assay. However, inhibition of receptor binding could not be confirmed on re-screening of the supernatants (Figure 5.4).

5.4. Discussion

Since the desired use of anti-receptor MAbs in this project was as receptor antagonists, it was important to screen for inhibition of binding to the ligand binding site. A receptor binding assay was therefore more relevant than screening assays such as immunoprecipitation or Western blotting. A major problem in the use of the receptor binding assay was the lack of positive controls. This is a common problem in the development of screening assays for hybridoma production. In the case of the ELISA for CGRP (Chapter 3), it was possible to optimize the assay using serum from immunized animals. This was not possible for the binding assay since no positive sera was detected using the assay. Some confidence in the assay, however, was gained when the reproducibility of the assay was assessed. The coefficient of variation of binding was low in the presence of relevant additives, and non-specific binding was also reproducible.

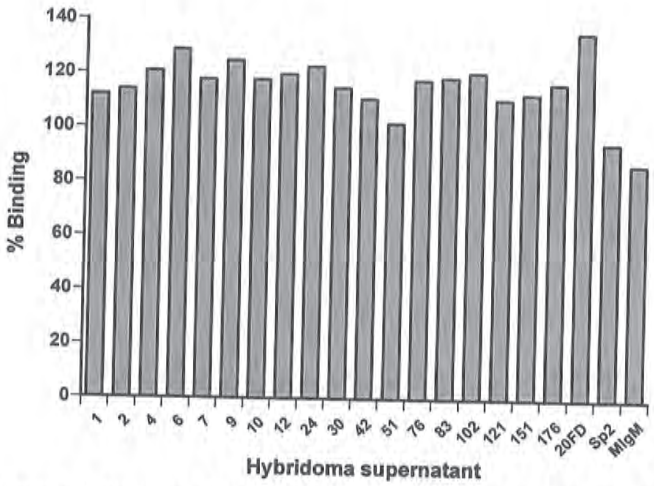

Figure 5.4: Screening of hybridoma supernatants from fusion following *in vitro* immunization with liver membrane preparation. The effect of supernatants on the specific binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to rat liver membranes is plotted. Controls were culture medium (20FD), Sp2 myeloma supernatant and non-specific mouse immunoglobulin (MIgG; 10 μg/ml). Values are the mean of two observations.

The variables investigated in the *in vivo* immunization protocols used in this study were: (1) amount of protein in the immunogen (1 to 20 mg), (2) tissue source of CGRP receptor, (3) species of receptor preparation and (4) species immunized. There are no definitive guidelines on immunization protocols for the generation of antibodies against large protein molecules. Microgram to 50 mg amounts of protein have been used by other workers (Goding, 1986). Assuming a molecular weight of 70 kDa for the CGRP receptor in rat liver (Chantry *et al.*, 1991; Stangl *et al.*, 1993), the amount of receptor (400 fmol/mg) in the crude immunogen used was only 2.8 x 10^{-5} times (0.003%) that of the membrane protein by weight. Nevertheless, MAbs against the insulin receptor have been generated by immunizing animals with whole IM-9 lymphocytes; the insulin receptor is estimated to represent less than 0.01% of a cell's membrane proteins (Roth & Morgan, 1985; Soos *et al.*, 1986). The cerebellum was used as an alternative source of receptors since it has been reported to be particularly rich in CGRP binding sites amongst tissues in the brain (Inagaki *et al.*, 1986; Henke *et al.*, 1987; Wimalawansa *et al.*, 1993). Bovine tissue was used since the potential sequence similarity of the mouse and rat receptor might prevent an effective immune response in the mouse. Due to known inter-animal variability in responses, relatively large numbers of mice and rats were immunized.

The purity of the immunogen *per se* is irrelevant in the production of MAbs. All that is required is a specific screening assay that would identify the antibody-secreting hybridoma of interest. However, impure material may give weaker specific responses. It is possible that contaminants could affect the response against the antigen of interest. Immunodominant antigens would give strong immune responses even when present in trace amounts. The responsiveness of individual animals to the various components of crude immunogens is reported to be highly variable, involving suppression as well as induction (Galfre & Milstein, 1981). When crude immunogens are used, it is critical that a highly specific and sensitive screening assay is available for screening. Crude membranes were used in this study for immunization under the assumption that the binding assay would serve as an effective screen. Failure to detect any inhibition of receptor binding by serum from mice immunized with the crude membranes suggested that success was unlikely. Therefore, no fusions were attempted.

The probability of raising anti-receptor antibodies would be increased if purified receptor was used for immunization and for the screening assay. However, immunization with purified receptor is not necessarily the perfect solution. Purified receptor may lose the native conformation and sub-components of the receptor moiety. Furthermore, antibodies will be generated in order of frequency according to the immunogenic potency of domains of the isolated receptor; these may not overlap with the functionally important domains *in situ*. Thus, an auto-anti-idiotypic approach (Chapter 6) was pursued in preference to the more conventional approach of immunizing with purified receptor.

In vitro immunization was used following unsuccessful *in vivo* immunization. Although *in vitro* immunization for the production of MAbs was reported as long ago as 1978, the technique has not been widely adopted. Nevertheless, this technique offered a number of theoretical and practical advantages over *in vivo* immunization which were relevant to the present study. *In vivo* immunization could have failed because of antigen-specific non-responsiveness (tolerance) or selective responsiveness to one or a few components of the immunogen preparation (antigen hierarchy response). In contrast, there is good evidence that tolerance and suppression could be broken by *in vitro* immunization and it has been possible to obtain antibodies against self or highly conserved antigens (Reading, 1982). Antibodies have been raised against soluble, membrane-bound or whole cell antigens using this technique (Reading, 1986). Amounts of antigen as low as nanograms have been used successfully. *In vitro* immunization is also rapid and has been claimed to lead to higher fusion frequencies with more hybrids secreting antibodies of interest (Boss, 1984). IgM antibodies tend to be produced by *in vitro* immunization. The size of the

IgM pentamer (900 kDa) is a potential disadvantage in that distribution of the large molecule may be restricted. However, it is possible to produce Fab fragments of IgM.

Coupling *in vitro* immunization with the standard fusion procedure in this study led to a fusion frequency of 100% with all hybrid lines secreting antibodies. Despite the success of the fusion, none of the hybridomas secreted anti-receptor immunoglobulins, as assessed by the receptor binding assay used.

In summary, no sera positive for receptor binding activity was found following *in vivo* immunization with crude membrane preparations. *In vitro* immunization was undertaken because the technique offered a number of potential advantages, but a successful fusion did not lead to hybridomas secreting supernatants with receptor binding activity.

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CHAPTER 6

Characterization of CGRP receptor binding of monoclonal antibodies raised by an auto-anti-idiotypic approach or by immunization with purified CGRP receptor

6.1. Introduction

The anti-idiotypic approach to the production of anti-receptor antibodies is based on the idiotypic-anti-idiotypic network hypothesis (Jerne, 1974; Jerne *et al.*, 1982). Idiotopes are antigenic determinants located in the variable regions of immunoglobulin molecules. The network hypothesis was formulated in recognition of the dual character of immunoglobulin molecules which consist of two distinct entities: combining site, or paratope, which interacts with antigens (epitopes), and idiotopes, which can be recognized by the paratopes carried on other antibody molecules. The anti-idiotypic response is heterogeneous and the network hypothesis attempts to classify the different kinds of idiotype-anti-idiotype interactions. The major criterion used to distinguish the different anti-idiotypic antibodies is the location of the target idiotope to which the anti-idiotype binds in relation to the antigen combining site (paratope). The location of the target idiotope can be mapped using the relevant antigen or hapten as the inhibitor in the binding of anti-idiotope to idiotope. If antigen no inhibition is observed, the target idiotope is assumed to be distant from the paratope, and perhaps located in a framework region (Bona & Kohler, 1984). Such antigen non-inhibitable anti-idiotypes are called Ab2α. If antigen inhibits the idiotope-anti-idiotope interaction, the target idiotope is believed to be in or near the paratope. Such anti-idiotypic antibodies, whose idiotopes cross-react with foreign epitopes, are postulated to bear the internal image of the original antigen and are classified as Ab2β. Other anti-idiotypes have been classified, for example, Ab2γ which are not internal image-bearing but nevertheless antigen-inhibitable through steric interference (Bona & Kohler, 1984).

The above classification of anti-idiotypes is probably simplistic and concepts are being revised (Kohler *et al.*, 1989; Erlanger, 1989). However, the concept of internal image bearing anti-idiotypic antibodies has offered the possibility of developing anti-receptor antibodies by immunizing with anti-ligand antibodies and, therefore, bypass the need to purify the receptor. The network hypothesis further predicts that immunization with a ligand would lead to anti-ligand antibodies (Ab1) which would, in turn, lead to anti-idiotypic antibodies (Ab2) in the same animal. It should be possible to screen for internal image-bearing Ab2β anti-idiotypes which mimic the ligand and bind to receptor.

The anti-idiotypic route to anti-receptor antibodies was first reported by Sege & Peterson (1978) who demonstrated that anti-idiotypic antibodies raised against antibodies to insulin could reproduce certain biological actions of the hormone itself upon binding to the insulin receptors of rat thymocytes. The findings were confirmed by Schechter *et al.* (1982) who also showed for the first time that immunization of mice with insulin led to the development of not only anti-insulin antibodies but also autologous anti-idiotypic anti-insulin receptor antibodies. These findings suggested that a normally functioning anti-idiotypic network exists and that there is an auto-anti-idiotypic route to anti-receptor antibodies.

Anti-receptor MAbs have been successfully generated by the two-step anti-idiotypic approach which consists of the isolation of an appropriate anti-ligand antibody followed by immunization with the purified anti-ligand antibody. A critical success factor for the two-step approach is that the anti-ligand antibody (Ab1) used for immunization should mimic the receptor as closely as possible. If an anti-idiotypic antibody that binds to the ligand binding site of the receptor is desired, an Ab1 that binds to the receptor binding site of the ligand must be identified. This is particularly difficult for macromolecular ligands that possess multiple epitopes, only one of which reacts with the receptor binding site. In practice, the identification of an Ab1 as a surrogate receptor can be time-consuming and is often left to chance. The generation of the appropriate Ab2 also relies on coupling of the ligand at a site that preserves its specificity for the receptor binding site. Despite the successes reported with the two-step approach (reviewed by Strosberg, 1989), accounts of failures have occasionally reached the literature, for example, the failure to generate anti-D_2 dopamine receptor antibodies (Abbott & Strange, 1986) and anti-aldosterone receptor antibodies (Lombes *et al.*, 1989a).

The feasibility of the one-step auto-anti-idiotypic approach to anti-receptor MAbs was first reported by Cleveland *et al.* (1983) who obtained anti-nicotinic acetylcholine receptor MAbs by immunizing mice with a structurally-constrained nicotinic receptor agonist. In addition to the lack of need to purify receptor, the one-step approach offers a further time-saving advantage that the choice of the appropriate anti-ligand antibody is left to the idiotypic network rather than the experimenter. Other successes with this approach include the development of MAbs against the glucocorticoid (Cayanis *et al.*, 1986), adenosine (Ku *et al.*, 1987), aldosterone (Lombes *et al.*, 1989b) and thyrotropin (Taub *et al.*, 1992) receptors.

The major objective of the present study was to screen MAbs raised by an auto-anti-idiotypic approach for CGRP receptor binding properties. The development of anti-CGRP receptor MAbs by the conventional approach of immunization with purified receptor was pursued by Wimalawansa (1992). The MAbs were initially

screened by ELISA with immobilized receptor purified from porcine cerebellum and had not been screened for receptor binding using rat or human tissues. Thus a further objective of the study was to screen these anti-porcine CGRP receptor MAbs for cross-reactivity with the rat and human CGRP receptors.

6.2. Methods

6.2.1. Source of potential anti-receptor MAbs studied

The MAbs studied in the present investigation come from two sources. Dr. C. Plumpton (Clinical Pharmacology Unit, Cambridge) provided 13 MAbs which were produced using an auto-anti-idiotypic approach. The MAbs were raised in mice which were immunized with RαCGRP (described in Chapter 3) and cloned twice on the basis that the antibodies do not bind RαCGRP but do bind affinity-purified rabbit PAbs against RαCGRP. These MAbs were coded Id1 to Id13 and were all isotyped as IgM. The Id MAbs used in the present study had been affinity purified by the use of an anti-mouse κ light chain MAb column.

Dr. S. Wimalawansa (Department of Medicine and Chemical Pathology, Royal Postgraduate Medical School, London) provided 5 MAbs which were raised against a CGRP receptor purified from porcine cerebellum (Wimalawansa, 1992; Wimalawansa *et al.,* 1993). These MAbs were coded with the prefix RCG. Hybridoma supernatants were used in the present study.

6.2.2. Receptor binding studies

Materials

2-[^{125}I]-iodohistidyl^{10}-HαCGRP	Specific activity 2000 Ci/mmol (Amersham)
SK-N-MC cells	ATCC No. HTB 10

Binding buffer (rat brain and SK-N-MC cells)[1]

Bacitracin	0.625 mg/ml
BSA	0.25%
$MgCl_2.6H_20$	5 mM
Tris HCl (pH 7.4)	50 mM

[1]The binding buffer used was similar to that used for the liver membrane binding assay with the omission of aprotinin and PMSF.

Wash buffer

$MgCl_2.6H_20$	5 mM
Tris HCl (pH 7.4)	50 mM

6.2.2.1. Experimental procedures

6.2.2.1.1. Rat liver membrane preparation

Rat liver membrane was prepared and binding assay performed according to the methods described in Chapter 5.

6.2.2.1.2. Rat whole brain membrane preparation

Eight Sprague-Dawley rats weighing approximately 300 g were stunned and guillotined. The whole brain was dissected out and kept in saline on ice. These were weighed in a 50 ml homogenizing tube and homogenized in approximately 50 ml buffer (50 mM Tris HCl pH 7.4, 5 mM $MgCl_2$). The homogenate was diluted to a total 20 volumes of buffer (i.e. 20 ml/kg), mixed on ice, and centrifuged at 48,000 *g* (20,000 rpm) at 4°C in a RC5C Sorvall Instruments (Dupont) centrifuge. The supernatant was discarded, the pellet resuspended in 20 volumes of buffer and the centrifugation repeated. The above step was repeated, giving 3 identical spins in all. The final resuspension was made in 2 volumes of buffer. One ml aliquots of the membrane suspension was stored at -20°C. Protein concentration was determined by the Biorad protein assay (see Chapter 4).

6.2.2.1.3. SK-N-MC human neuroblastoma cell membrane preparation

SK-N-MC cells were harvested, spun down and the pellet weighed (approximately 0.25 g). Cells were then homogenized with 20 strokes of a glass-Teflon homogenizer at 650 rpm in 20 volumes (i.e. 20 ml/g) of 5 mM Tris HCl pH 7.4. The homogenate was centrifuged at 48,000 *g* (20,000 rpm) for 20 minutes at 4°C in a RC5C Sorvall centrifuge. The supernatant was discarded, the pellet resuspended as above in 20 volumes of 5 mM Tris HCl pH 7.4 and left on ice for 1 hour to allow cell lysis. The above spin was then repeated. The pellet was resuspended in 20 volumes of buffer (50 mM Tris HCl pH 7.4, 5 mM $MgCl_2$) and the membrane preparation stored in aliquots at -20°C.

6.2.2.1.4. Binding assay (rat whole brain or SK-N-MC cell membrane preparation)

Whatman GF/C filters were soaked in 0.5% polyethyleneimine for at least 2 hours prior to filtering. The appropriate volume of rat brain membrane suspension was thawed, re-homogenized using a glass-Teflon homogenizer, and diluted to 2.5 mg/ml with 50 mM Tris HCl buffer pH 7.4 containing 5 mM $MgCl_2$. SK-N-MC cell membranes were thawed, re-homogenized and used undiluted (approximately 100-200 µg protein per tube).

The assay volume was 250 µl . Twenty-five µl of unlabelled CGRP was added to each tube to give final concentrations of 10^{-12} to 10^{-7} M. Tubes for total binding contained assay buffer only. Non-specific binding tubes contained 10^{-7} M of unlabelled CGRP. Twenty-five µl of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP (approximately 36,000 cpm) was added to each tube to give a final concentration of 40 pM. The appropriate volume of assay buffer was added. One hundred µl of membrane suspension (250 µg protein for rat brain) was added to each tube. The tubes were vortex mixed and the assay mixture incubated for 60 minutes at room temperature.

For the screening of antibodies, 50 µl Id MAb solutions were added to the polypropylene tubes (Starstedt) to give final concentrations of 15 to 60 µg/ml. Fifty µl RCG MAb culture supernatants were tested. In some experiments MAbs were incubated with membranes for 5 days at 4 °C prior to addition of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP and further incubation for 1 hour at room temperature. No difference in total and non-specific binding was observed after the 5 day incubation of the membrane suspension at 4°C compared with freshly thawed membrane suspension.

Samples were filtered through the pre-soaked GF/C filters on a Brandel 24-well cell harvester, followed by three washes with 3 ml ice-cold wash buffer. Filter paper was placed into counting tubes and counted for 1 minute in a LKB Wallac 1272 Clinigamma counter with four 1.5 inch detectors.

6.2.3. Immunocytochemistry

6.2.3.1. Principles

The immunocytochemical method used in the present study was based on the Avidin-Biotin Complex (so-called ABC) system. The immunoperoxidase staining technique employed an unlabelled primary antibody, followed by a biotinylated secondary antibody and then a pre-formed avidin and biotinylated horseradish peroxidase macromolecular complex.

Avidin is a large glycoprotein from egg white which has a very high affinity (four binding sites per molecule) for biotin, a vitamin of low molecular weight found in egg yolk. Biotin can be coupled to antibody in high molecular proportion, or to a label such as peroxidase. Avidin may also be labelled with, for example, peroxidase or fluorescein. The complex of avidin and biotinylated peroxidase are reacted together in such proportion that some biotin-binding sites on the avidin molecule are not filled by biotinylated peroxidase, but are free to react with the biotin of the second antibody.

The substitution for avidin with streptavidin, derived from *Streptococcus avidini*, offers some advantages. The streptavidin molecule is uncharged relative to animal tissue, unlike avidin which has an isoelectric point of 10, and therefore electrostatic binding to tissue is eliminated. In addition, streptavidin does not contain carbohydrate groups which might bind to tissue lectins. A streptavidin-biotinylated horseradish peroxidase complex was evaluated in the present study.

The Vectastain® *Elite* ABC kit eventually chosen in the present study contained Avidin DH and biotinylated horseradish peroxidase H reagents which have been prepared to form ideal complexes for immunoperoxidase staining.

Materials	**Supplier**
Normal horse serum	Vector Laboratories, Peterborough
Anti-mouse κ light chain MAb 187.1 (biotinylated by C. Plumpton)	European Collection of Animal Cell Cultures, Porton Down
Biotinylated horse anti-mouse IgG (H + L; rat adsorbed; BA-2001)	Vector
Purified mouse IgM, κ from TEPC tumour line (M-2770)	Sigma
Affinity-purified rabbit anti-CGRP PAbs	C. Plumpton
Streptavidin-biotinylated horseradish peroxidase complex (RPN1051)	Amersham
Fluorescein-streptavidin (RPN 1232)	Amersham
Vectastain® *Elite* ABC kit	Vector
3,3' Diaminobenzidine (DAB)	Amersham
Hydrogen peroxide solution 30%	BDH
Paraformaldehyde	BDH
Xylene	BDH
DEPEX mounting medium[2]	BDH
Propidium iodide	Molecular Probes
CitiFluor aqueous mountant	City University, London
Triton-X 100 (10% stock in PBS)	Sigma

[2]DEPEX is named for its components, 10 g distrene 80, 5 ml dibutyl phthalate, and 35 ml of xylene

Reagents

0.2 M Phosphate buffer

Solution A: 31.2 g sodium dihydrogen orthophosphate in 1000 mls of deionized H_20.

Solution B: 28.3 g disodium hydrogen orthophosphate in 1000 mls of deionized H_20.

9.5 ml of A + 405 ml of B, made up to 500 ml with deionized H_20.

4% Paraformaldehyde

Paraformaldehyde	80 g
Deionized H_20	500 ml

(Heated with stirring to 60°C, held for 5 minutes, and 1 M NaOH added dropwise to clear solution.)

The above solution was cooled and added to 500 ml 0.2 M phosphate buffer. The pH of the solution was adjusted to 7.4 with HCl.

Peroxidase substrate solution (prepared just prior to use)

DAB	25 mg
Hydrogen peroxide (30%; 100 volumes)	16 µl (just prior to use)
0.1 M PBS/0.3% Triton-X 100 (PBSTx)	100 ml

Vectastain® Elite ABC reagent

Reagent A	2 drops (from bottle supplied)
Reagent B	2 drops
0.1 M PBS pH 7.4	5 ml

The solution was mixed immediately and allowed to stand for 30 minutes prior to use.

Gelatin-subbing of slides

Glass slides were immersed in Decon detergent solution overnight, rinsed and dried in an oven. Six grams of gelatin was dissolved in 1200 ml deionized water by stirring and heat. When dissolved, 0.6 g of chromic potassium sulphate was added and slides immersed in this solution for 5 minutes. Slides were then dried in an oven.

6.2.3.2. Transcardiac perfusion fixation

Fixation protocols must (1) prevent antigen leakage, (2) permeabilize the cell to allow access of the antibody, (3) keep the antigen in such a form that it can be recognized efficiently by the antibody, and (4) maintain the cell structure. Transcardiac perfusion is the method of choice for the preservation of central nervous system tissue. It aids fixation by allowing excellent and quick penetration of fixative and thus prevents a number of artefacts associated with the far slower immersion fixation of tissues. Paraformaldehyde is a cross-linking reagent which form intermolecular bridges,

normally through free amino groups, thus creating a network of linked antigens. However, such fixation may denature protein antigens.

6.2.3.2.1. Experimental procedures

Each Sprague-Dawley rat was deeply anaesthetized by i.p. injection of pentobarbitone sodium (Rhone-Merieux). The chest cavity was opened to expose the heart. The descending aorta was clamped and the perfusion needle inserted at the apex of the heart into the left ventricle. Once the needle was inserted, the right atrium was cut to allow irrigation of the upper vasculature of the animal. The rat was perfused with 0.9% saline containing 25 units/ml heparin at 100 mmHg pressure (approximately circulation pressure) until the fluid leaving the atrium appeared clear (approximately 300 ml). This was followed by perfusion with freshly prepared 4% paraformaldehyde in 0.1 M PBS pH 7.4 until the upper body of the animal appeared pale and was rigid (approximately 700 ml). The entire spinal column was then dissected and immersed in 4% paraformaldehyde at 4°C overnight. The spinal cord was removed the next day and immersed overnight in 30% sucrose in 0.1 M PBS pH 7.4 at 4°C.

6.2.3.3. Snap freezing of tissues

It is important that tissue is frozen rapidly to prevent the formation of ice crystals (from water within the tissue) which will damage the tissue. Tissues were snap-frozen in isopentane at -35 to -40°C. This was achieved by cooling a beaker of isopentane on dry ice and monitoring the temperature of isopentane with a temperature probe. At this temperature, the white and grey matter of tissues from the central nervous system freeze at the same rate, thus minimizing any cracking of tissue. Frozen tissue were used fresh or stored at -70°C

6.2.3.4. Cryostat sections

In experiments involving the use of free-floating sections, 30 μm sections were cut from the rat spinal cord (L4-L5) at -20°C using a 2800 Frigocut E cryostat (Reichert-Jung) or a Bright OTF/AS cryostat (Bright Instrument, Huntingdon). Sections were immediately immersed in 0.1 M PBS/0.3% Triton-X 100 (PBSTx) in permeable plastic capsules (Agar Scientific Ltd., Stansted) placed within the wells of a 24-well tissue culture plate (CoStar). Two spinal cord sections were placed into each well.

6.2.3.5. Immunocytochemical staining of free-floating tissue sections

6.2.3.5.1. Optimization of staining procedure

The immunostaining procedures described below were optimized by systematically investigating the effect of several experimental variables on the staining of adjacent tissue sections. The optimal concentration of primary antibody was determined by dilution studies. Two secondary antibodies were compared: biotinylated anti-mouse κ light chain MAb 187.1 and the Vector biotinylated horse rat-absorbed anti-mouse IgG which exhibits cross-reactivity with mouse IgM. Streptavidin-biotinylated horseradish peroxidase complex from Amersham (diluted 1 in 200 with PBSTx) and the Vectastain® *Elite* ABC reagent were compared. The concentration of DAB was compared at 0.025% and 0.05%.

6.2.3.5.2. Experimental procedures

All procedures were carried out at room temperature with the exception of overnight incubation with primary antibody at 4°C. Incubation and washing steps were performed with gentle shaking of the tissue culture plate on an orbital tray shaker. The washing step consisted of reagent removal followed by 3 incubations with 300 µl fresh PBSTx over 5 minute intervals.

PBSTx was removed from wells containing free-floating sections by the use of a Pasteur pipette attached to a vacuum. Four hundred µl of 0.1 M PBS containing normal horse serum 3%, 0.1% BSA and 0.3% Triton-X 100 (blocking reagent) was added to each well. Tissue sections were incubated with blocking reagent for 1 hour. After removal of the blocking reagent, tissue sections were incubated overnight at 4°C with 300 µl of primary antibody appropriately diluted in blocking reagent (15 to 60 µg/ml for Id MAbs). RCG MAb supernatants were tested undiluted. Tissue sections were washed and incubated for 90 minutes with 300 µl of biotinylated secondary anti-mouse antibody at a dilution of 1 in 200 (in PBSTx). This was followed by washing and incubation for 60 minutes with 300µl of Vectastain® *Elite* ABC reagent. After washing, 300 µl substrate solution (DAB solution 0.025% containing 0.005% hydrogen peroxide in PBSTx) was added and the development of colour monitored under a microscope (Wild Heerbrugg; Leitz Instruments Ltd.). The reaction was stopped by washing twice in deionized water.

Tissue sections were mounted on glass slides (non-gelatinized; Chance Propper Ltd., Smethwick, Warley) with the aid of a brush and allowed to air dry. Slides were sequentially immersed in 70% ethanol (5 minutes), absolute ethanol (5 minutes), and finally in xylene (5 minutes). Coverslips (Chance Propper Ltd) were mounted with the non-aqueous mounting medium DEPEX.

6.2.3.5.3. Experimental controls

Method specificity was determined by the use of omission controls. Primary antibody, biotinylated secondary antibody, streptavidin-biotinylated horseradish peroxidase complex and DAB were omitted in control tissue sections in order to identify the source of potential non-specificity.

Antibody specificity was investigated by the use of a non-specific mouse IgM as a control antibody and testing whether pre-absorption with antigen could diminish immunostaining. Having determined the lowest concentration of primary antibody compatible with good immunostaining, Id MAbs were pre-absorbed with affinity-purified rabbit anti-CGRP PAbs (10 to 50 times excess molar concentration) overnight prior to use in immunostaining of tissue sections.

The most relevant question in the present study was whether immunostaining with Id MAbs could be attributable to CGRP receptor localization. CGRP itself should inhibit immunostaining if CGRP receptors were localized by Id MAbs. Therefore, tissue sections were pre-incubated with excess RαCGRP (10 μM) in binding assay buffer or binding assay buffer alone, blocked with normal horse serum (blocking reagent above), followed by overnight co-incubation of Id MAbs with 10 μM RαCGRP.

6.2.3.6. Immunocytochemistry using fresh (unfixed) tissue sections

The immunostaining of fresh, unfixed, spinal sections was tested because of the possibility that paraformaldehyde fixation denatures the CGRP receptor. If immunostaining of fresh tissue sections was possible, it would allow testing of the specificity of Id MAbs for the CGRP receptor by displacement with excess CGRP.

The immunocytochemical procedure used was similar to that described above for fixed tissue sections. However, tissue sections were mounted on slides and thinner 14 μm sections were used to facilitate antibody penetration. In addition, Triton-X 100 was avoided in all steps.

6.2.3.7. Immunocytochemistry of cultured cells

Immunostaining of live and fixed cells was attempted in order to answer the major question of whether Id and RCG MAbs specifically localize the CGRP receptor. Specificity could be assessed by studying cells previously shown by receptor binding studies to be positive or negative for CGRP receptors. Immunostaining live cells would avoid the potential denaturing effect of paraformaldehyde fixation and allow the performance of displacement studies with CGRP. In addition, higher resolution

may be obtained and 3-dimensional views obtained by confocal fluorescence microscopy could identify whether a membrane protein is immunostained (e.g., see Ornatowska & Glasel, 1992).

The SK-N-MC human cell line clearly expresses CGRP receptors, as shown in the present study and by others (Semark *et al.*, 1992). However, specific CGRP binding could not be demonstrated in a related cell line, SK-N-SH. This cell line was therefore used as a negative control. A major interest was to use anti-receptor MAbs as pharmacological tools in the rat. Therefore, the rat L6 myocyte cell line which expresses high affinity CGRP receptors was investigated. Specific binding of CGRP to intact L6 myocytes has been demonstrated (Poyner *et al.*, 1992). The AR42J rat exocrine pancreas cell line which does not express CGRP receptors (Poyner *et al.*, 1992) was used as a negative control.

6.2.3.7.1. Cell culture

SK-N-MC (ATCC No. HTB 10) and SK-N-SH (ATCC No. HTB 11) human neuroblastoma cells were obtained from the American Type Culture Collection. L6 rat thigh muscle cells (ECACC No. 85011421) and AR42J rat exocrine pancreas cells (ATCC No. CRL 1492) were obtained from the European Collection of Animal Cell Cultures and Flow Laboratories respectively. Cells were cultured in media specified by the suppliers and grown to about 80% confluence for immunostaining.

6.2.3.7.2. Coating of coverslips with Poly-L-lysine

Cells were grown on poly-L-lysine coated coverslips within wells of 12-well tissue culture plates for the purpose of immunostaining. Poly-L-lysine binds to most solid supports through its charged side chains. The positively charged polymer provides a site for binding of cells (which carry a overall negative charge). Although this cross-link is not covalent, it is sufficiently strong for most cell staining techniques.

The following procedures were performed in a laminar flow cabinet. Poly-L-lysine (Sigma) was dissolved in sterile water to 5 μg/ml. Coverslips (18 mm diameter) were placed into wells of a 12-well tissue culture plate. One ml poly-L-lysine was added to each well. After an incubation period of 30 minutes, the solution was aspirated and coverslips washed with 1 ml sterile water. The coverslips were allowed to dry before the seeding of cells.

6.2.3.7.3 Experimental procedures

The immunostaining procedures were performed in 12-well tissue culture plates at room temperature. Except for the final wash, each washing step consisted of reagent removal followed by 3 incubations with fresh 0.1 M PBS over 5 minute intervals.

Cells were removed from the incubator, washed in DMEM medium (with 25 mM HEPES and 4.5 g glucose but without pyruvate [Gibco]) for 5 minutes, followed by incubation in the same medium containing 3% normal horse serum (blocking reagent) for 60 minutes. The blocking reagent was removed and cells incubated with Id MAbs diluted in blocking reagent (15 to 60 μg/ml) for 60 minutes. RCG MAb supernatants were tested undiluted. A control without primary antibody was included. After washing, cells were incubated with biotinylated anti-mouse secondary antibody (Vector) diluted 1 in 200 in 0.1 M PBS for 60 minutes. The cells were washed and incubated for 60 minutes with fluorescein-streptavidin diluted 1 in 200 with 0.1 M PBS. After a final wash in PBSTx, cells were incubated with the nuclear counterstain propidium iodide (1 mg/ml stock; diluted 1 in 5000 with PBSTx) for 30 seconds. A small drop of aqueous mountant (CitiFluor) was added to glass slides for the mounting of coverslips. A coverslip with adhered cells was gently apposed to the aqueous mountant on a glass slide and allowed to dry for a short period. Finally, the coverslip was sealed and fixed in position with clear nail varnish.

Immunostaining of fixed cells was also performed. Fixation was performed by washing cells with 0.1 M PBS followed by incubation with 4% paraformaldehyde in 0.1 M PBS for 10 minutes. After washing, the same procedures described above were followed.

Immunoperoxidase staining using the same procedures described for tissue sections was also attempted.

6.2.3.8. Microscopy and Photography

Tissue sections were viewed under a transmitted light microscope (Leitz Dialux 20EB, Germany). Photography was performed a Wild Photoautomat MPS 51S/45 camera attached to the microscope. A rare earth dydidium filter was placed over the light source and the light condenser was used except in lowest power. EPT-160T (Kodak Ektachrome; 160ASA, tungsten) slide film was used.

For immunofluorescence experiments, a drop of immersion oil was placed on top of the coverslip and the slide viewed under a fluorescence microscope (Leitz; Filter 1; the wavelength for maximal excitation of fluorescein is 495 nm).

6.2.3.9. Image Analysis

Image analysis of Id MAb immunostained sections was performed to assess whether the intensity of cell staining corresponded to the expected distribution of receptors as assessed by receptor autoradiography.

The MCID image analysis system (Microcomputer Imaging Device; Imaging Research Inc., Brock University, Ontario, Canada) was used. The system consisted of a PC-compatible computer running under the OS/2 operating system, an imaging system and software. A Compaq Deskpro *386/20e* computer with a 80387 co-processor was used. The imaging system consisted of the MCID imaging board, shielded RGB monitor, Cohu solid state camera, Micro Nikkor 55mm lens with video adaptor and extension tubes, Northern light illuminator and Kaiser RS1 copy stand. Prior to analysis, the system was calibrated by establishing the shading error in the middle of the dynamic range of the system. Hard copy images were obtained using a colour video printer (Sony UP-5000P).

6.2.4. Receptor autoradiography

The main objective of the receptor autoradiography experiment was to assess whether paraformaldehyde fixation leads to the denaturation of CGRP receptors in the spinal cord. Therefore, autoradiography was performed using fresh and perfusion-fixed spinal cord sections. A secondary objective was to compare the immunostaining patterns in spinal cord sections, as assessed by image analysis, with the autoradiographic distribution of CGRP receptors.

6.2.4.1. Principles

The rationale for various procedures used in receptor autoradiography have been reviewed by Kuhar (1985). Pre-incubation of tissue sections with buffer has been shown to enhance receptor binding, possibly by leaching the endogenous ligand from tissues. The ligand incubation period should be optimized to allow maximal ligand association with minimal enzymatic degradation of the ligand. The purpose of the washing step is to reduce non-specific binding more than specific binding. A wash time is selected such that a workable specific:non-specific ratio is obtained while most specific binding is maintained intact. A final rinse in deionized water is performed to remove buffer salts which can cause autoradiographic artefacts.

6.2.4.2. Experimental procedures

Fresh cryostat sections (12 μm) were thaw-mounted on gelatin-subbed glass slides and dried in a stream of air for 60 minutes. Tissue sections were stored at -70°C with desiccant when necessary. Frozen sections were thawed and also dried in a stream of air. The entire binding procedure was performed at room temperature. The binding buffer used for receptor autoradiography was identical to the buffer used in the receptor binding assay described for the rat brain membrane preparation.

Following a 15 minute pre-incubation in 50 mM Tris HCl buffer containing 5 mM $MgCl_2$, tissue sections were incubated with 50 pM 2-[^{125}I]-iodohistidyl10-HαCGRP (approximately 80,000 cpm in 50 μl) for 90 minutes for the determination of total binding. Non-specific binding was determined by co-incubation with 10 μM RαCGRP. The tissue sections were washed twice for 5 minutes in assay buffer followed by a final rinse in deionized water to remove salts. Tissue sections were dried under a stream of cool air for 20 minutes. Slides were then secured with double-sided tape on to an X-ray cassette. In a darkroom, a sheet of Betamax Hyperfilm (Amersham) was apposed to the set of slides with the emulsion side on slides. The cassette was closed, sealed and stored for until development 7 days later.

The autoradiograph was developed by immersion in developer solution (Kodak HC-110) for 5 minutes, stop solution (Ilford IN-1 stop bath) for 1 minute and fixing solution (Ilford HYPAM containing Ilford RAPD hardener) for 5 minutes. The solutions were diluted according to manufacturers' directions. Each tissue section was counter-stained with cresyl violet to reveal the cellular pattern.

6.2.5. Enzyme-linked immunoadsorbent assay (ELISA)

The indirect ELISA used was identical to the procedures described in Chapter 3 except for the use of immunoglobulins as coating antigens. The following materials and procedures were specific to the ELISAs used for the present investigations.

Materials	Supplier
Rabbit PAbs against glial fibrillary acidic protein	DAKO
Purified normal rat IgG	Sigma
Rat-absorbed goat anti-mouse Fab-specific biotin conjugate (B-0529)	Sigma
Horseradish peroxidase-streptavidin (SA-5004)	Vector
Biotinylated goat anti-mouse IgM (μ chain specific; BA-2020)	Vector
DEAE-purified rabbit non-immune IgG	C. Plumpton

6.2.5.1. ELISAs to investigate the "internal image" property of Id MAbs

An ELISA was performed to assess whether the binding of Id MAbs to affinity-purified rabbit anti-CGRP PAbs could be inhibited by co-incubation with excess RαCGRP. The protocol has been successfully used for the screening of internal image-bearing anti-idiotypic antibodies (Farid, 1989). An ELISA plate was coated with 1.5 μg/ml affinity-purified rabbit anti-CGRP PAbs (approximately 10 nM IgG). After blocking and washing steps, Id MAb supernatants were added with 1 μM Rα CGRP (final concentration) or vehicle and co-incubated for 2 hours at room temperature. The remaining procedures were as described for the ELISA in Chapter 3.

6.2.5.2. ELISAs to investigate the potential anti-immunoglobulin binding of Id MAbs

Initially an irrelevant affinity-purified rabbit PAb preparation against glial fibrillary acidic protein (1.5 μg/ml) was used as a control to coat ELISA plates. The finding that Id MAbs bound to these PAbs as well as affinity-purified rabbit anti-CGRP PAbs prompted a further experiment in which ELISA plates were coated with DEAE-purified non-immune rabbit IgG (1.5 μg/ml).

In order to investigate whether the immunostaining detected using Id MAbs in rat spinal cord sections could be attributed to binding to IgG present on cell surfaces, ELISA plates were coated with rat IgG (1.5 μg/ml; Sigma) and the assay performed using rat-absorbed goat anti-mouse Fab-specific biotin conjugate (Sigma) and horseradish peroxidase-streptavidin (Vector).

An experiment was also performed to investigate whether the cross-reactivity with immunoglobulins from different species was because the Id MAbs were actually directed to epitopes on the Fc fragment of mouse immunoglobulin. ELISA plates were coated with 10 μg/ml of $F(ab')_2$ or IgG of the anti-CGRP mouse MAb R1.50. Since the Id MAbs were of the IgM subclass, the assay was performed using biotinylated goat anti-mouse IgM (Vector).

Sp2 myeloma supernatant and non-specific mouse IgM (10 μg/ml) were used as controls in the above experiments.

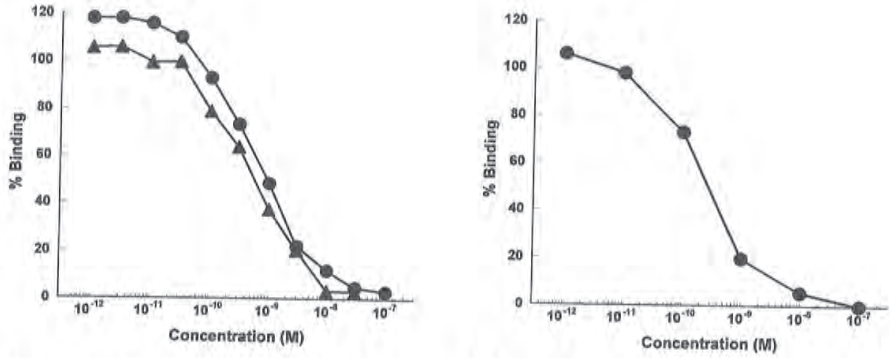

Figure 6.1: Displacement of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP binding to rat brain membrane preparation (a) or to SK-N-MC cell membrane preparation (b) by RαCGRP (circles) or HαCGRP (triangles). Values are the mean of 2 observations.

6.3. Results

6.3.1. Auto-anti-idiotypic approach: Id MAbs

6.3.1.1. Receptor binding studies

The specific displacement of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP binding to rat brain and SK-N-MC cell membranes by RαCGRP and HαCGRP was confirmed (Figure 6.1). However, MAbs Id1 to Id13, at 15 to 60 μg/ml, did not inhibit the binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to rat liver, rat brain and SK-N-MC cell membrane preparations (Figure 6.2).

6.3.1.2. Immunocytochemistry and receptor autoradiography

Several Id MAbs (1, 3, 4, 5, 11, 12), but not the non-specific IgM, immunostained cells in rat spinal cord sections (for example MAb Id1; Figure 6.3). The staining was diminished by pre-incubation of MAbs with excess affinity-purified rabbit anti-CGRP PAbs. However, background staining was high in the sections co-incubated with the PAbs, probably because of the high PAb concentrations used and some cross-reactivity of the secondary anti-mouse antibody with rabbit antibody. Pre-incubation and co-incubation with a high concentration of RαCGRP (10 μM) did not affect immunostaining (for example, Id3; Figure 6.4).

Image analysis of the immunostained sections (Figure 6.5) suggested some similarity in the distribution of cell staining and the autoradiographic distribution of CGRP binding sites (Figure 6.6; see also Garry *et al.*, 1991; Yashpal *et al.*, 1992). The most striking feature of the autoradiographic distribution of CGRP binding sites was a

U-shaped band that included lamina X (around central canal) and the medial parts of lamina III to IV. Low density of CGRP binding sites was observed in the ventral horn. The density of immunostaining generally corresponded to the autoradiographic picture.

Comparison of the binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to unfixed and paraforrmaldehyde-fixed spinal cord sections (Figures 6.6 and 6.7) showed that fixation denatured CGRP binding sites. Unlike fresh tissue sections, total binding was almost homogeneously distributed in paraformaldehyde-fixed sections and non-specific binding in the presence of 10 μM RαCGRP was high. This experiment showed that it was not possible to investigate the specific inhibition of immunostaining by Id MAbs with excess CGRP. However, immunostaining of unfixed, slide mounted, tissue sections was found to be possible (Figure 6.8).

None of the Id MAbs immunostained live or fixed SK-N-MC or L6 cells which were known to express CGRP receptors.

6.3.1.3. ELISAs

Co-incubation of Id MAbs with RαCGRP did not inhibit the binding of Id MAbs to rabbit affinity-purified anti-CGRP PAbs by indirect ELISA (Figure 6.9).

Id MAbs bound not only to anti-CGRP rabbit PAbs by ELISA, but also to irrelevant affinity-purified rabbit PAbs against glial fibrillary acidic protein and DEAE-purified non-immune rabbit IgG. This was the first indication that the Id MAbs were simply anti-immunoglobulin antibodies. These MAbs also bound to rat IgG. Positive ELISA results were obtained for mouse MAb R1.50 IgG but not F(ab')$_2$ fragments (Figure 6.10).

6.3.2. Anti-receptor MAbs: RCG MAbs

The RCG MAbs did not inhibit the binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to human neuroblastoma SK-N-MC cell or rat brain membrane preparations (Figure 6.11). No specific immunostaining was detected with these MAbs in spinal cord sections or in culture cells whether live or fixed.

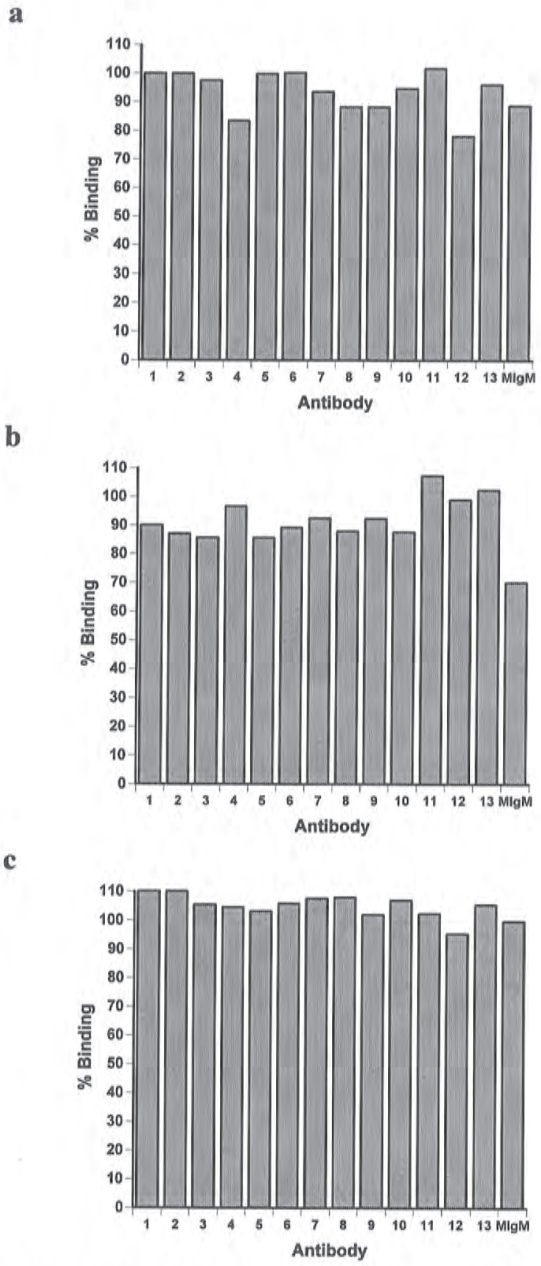

Figure 6.2: Effect of MAb Id1 to Id13 and non-specific mouse IgM (MIgM) on the binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to rat liver (a), rat brain (b) and human neuroblastoma SK-N-MC cell (c) membrane preparations. Specific binding is expressed as a percentage of vehicle control. Each value is the mean of two observations.

U-shaped band that included lamina X (around central canal) and the medial parts of lamina III to IV. Low density of CGRP binding sites was observed in the ventral horn. The density of immunostaining generally corresponded to the autoradiographic picture.

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Id MAbs bound not only to anti-CGRP rabbit PAbs by ELISA, but also to irrelevant affinity-purified rabbit PAbs against glial fibrillary acidic protein and DEAE-purified non-immune rabbit IgG. This was the first indication that the Id MAbs were simply anti-immunoglobulin antibodies. These MAbs also bound to rat IgG. Positive ELISA results were obtained for mouse MAb R1.50 IgG but not F(ab')$_2$ fragments (Figure 6.10).

6.3.2. Anti-receptor MAbs: RCG MAbs

The RCG MAbs did not inhibit the binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to human neuroblastoma SK-N-MC cell or rat brain membrane preparations (Figure 6.11). No specific immunostaining was detected with these MAbs in spinal cord sections or in culture cells whether live or fixed.

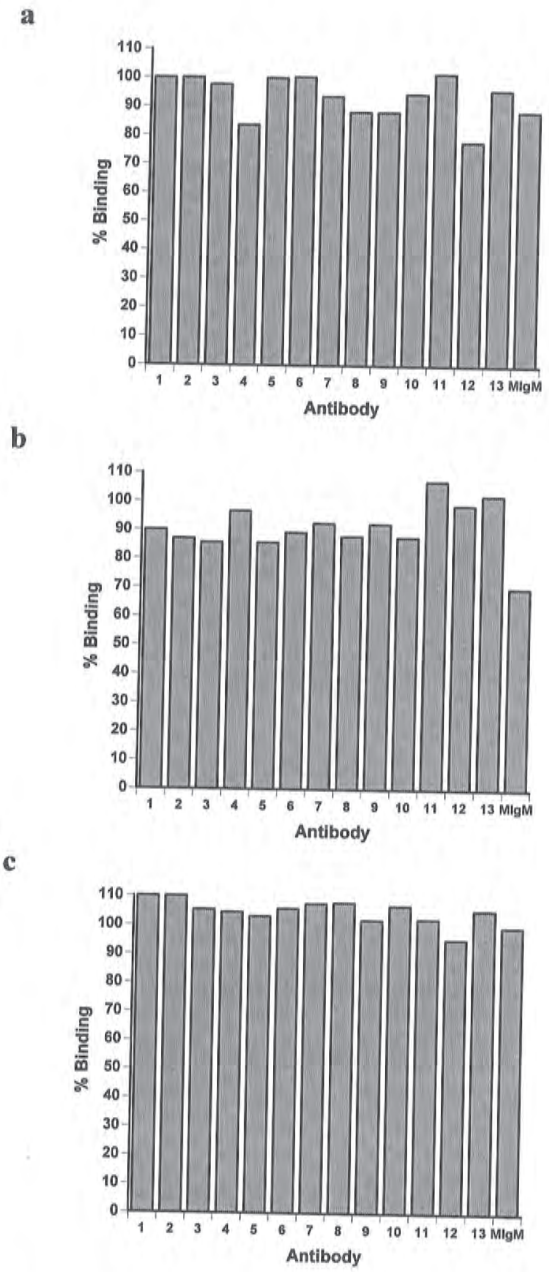

Figure 6.2: Effect of MAb Id1 to Id13 and non-specific mouse IgM (MIgM) on the binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to rat liver (a), rat brain (b) and human neuroblastoma SK-N-MC cell (c) membrane preparations. Specific binding is expressed as a percentage of vehicle control. Each value is the mean of two observations.

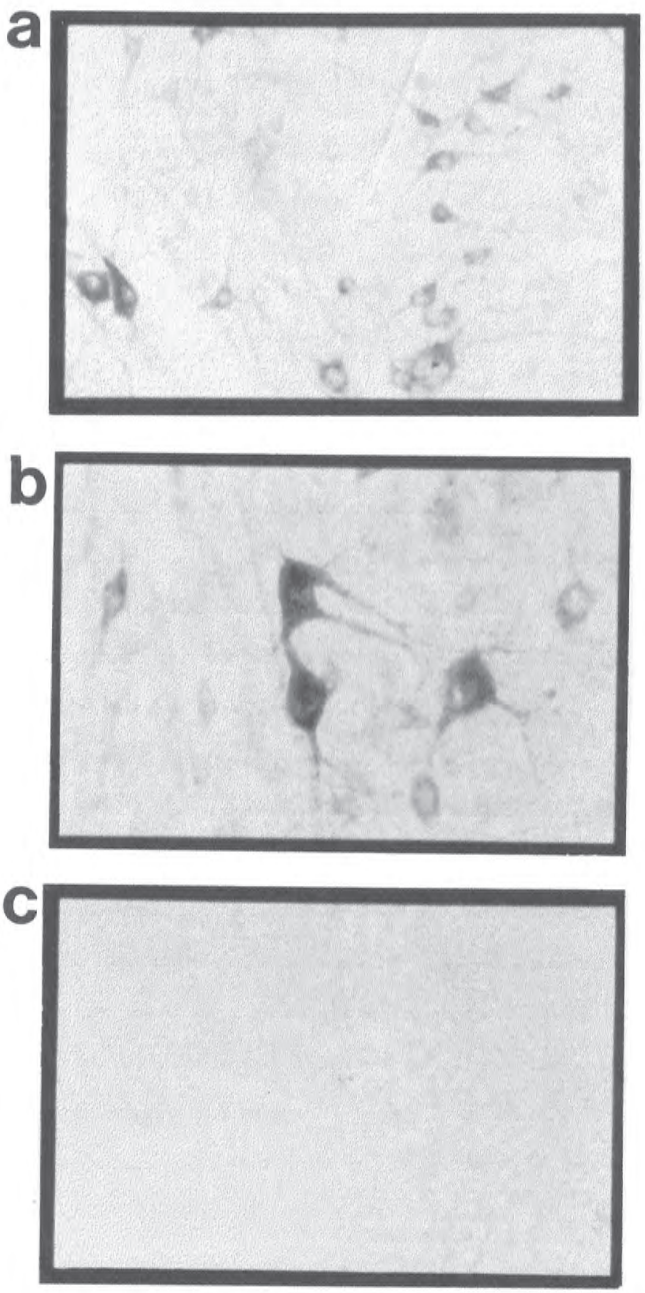

Figure 6.3: Immunostaining of cells by MAb Id1 (30 μg/ml) in the grey matter (a; ventral horn; x250) and white matter (b; x400) of the rat spinal cord. No staining was obtained by the use of a non-specific mouse IgM at 30 μg/ml (c; ventral horn; x250).

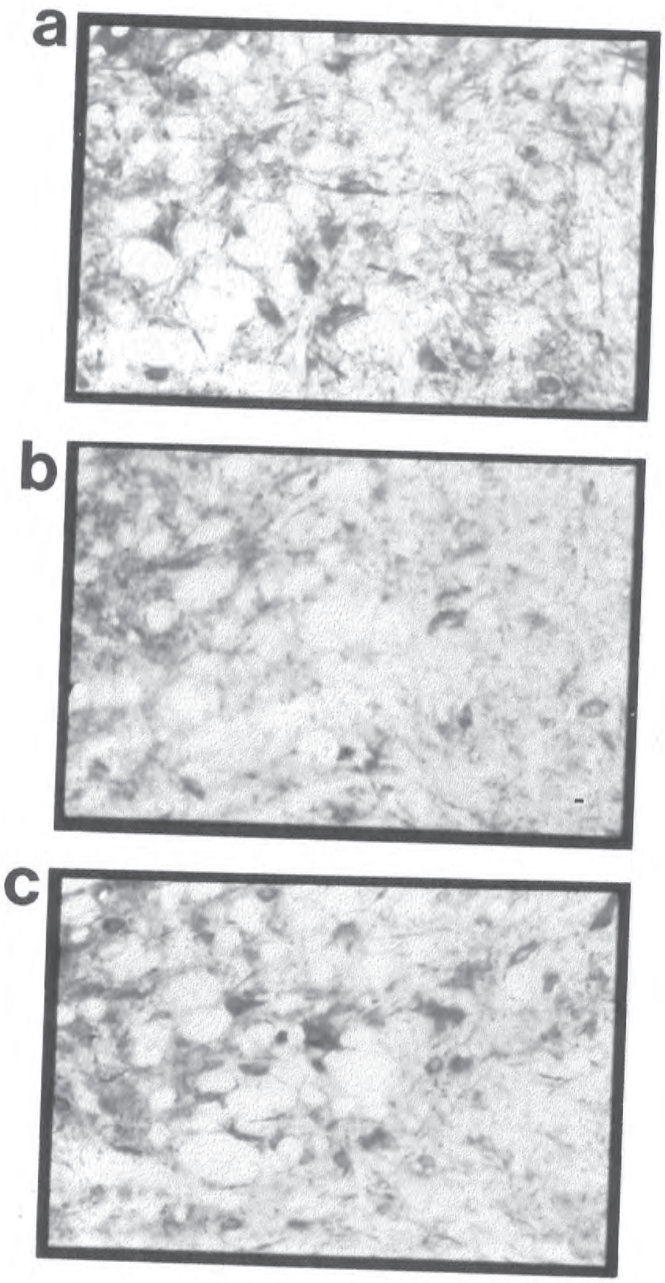

Figure 6.4: Immunostaining by MAb Id3 at 30 μg/ml (a; ventral horn; x250) was diminished by pre-incubation of the MAb with 800 μg/ml affinity-purified rabbit anti-CGRP PAbs (b) but not with 10 μM RαCGRP (c).

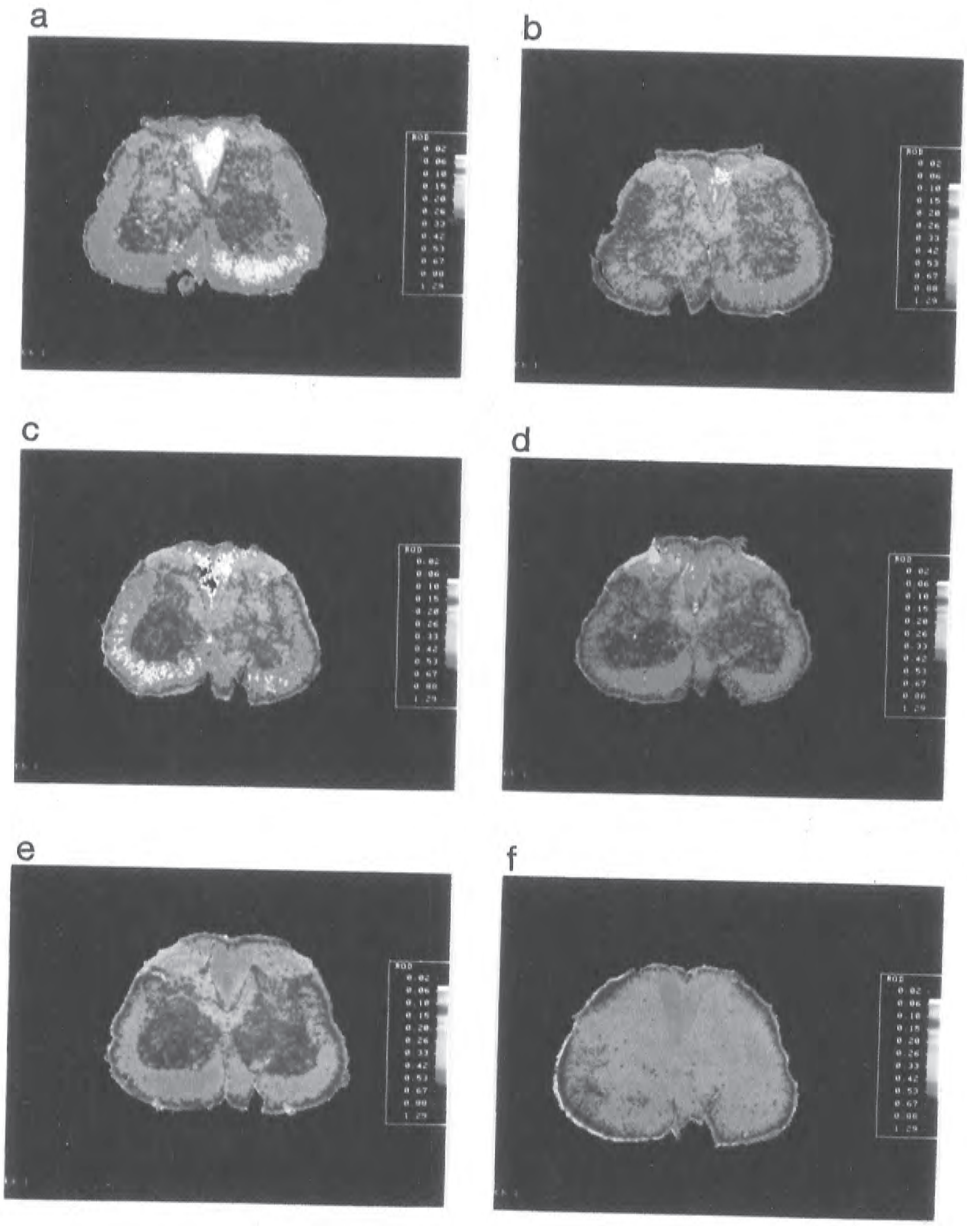

Figure 6.5: Image analysis of whole spinal cord sections immunostained with MAbs Id1 (a), Id5 (b), Id4 (c), Id11 (d), Id12 (e) and non-specific IgM (f). The colour coding for relative optical density is shown on the left of each figure. The concentration of MAbs used was 30 to 60 μg/ml.

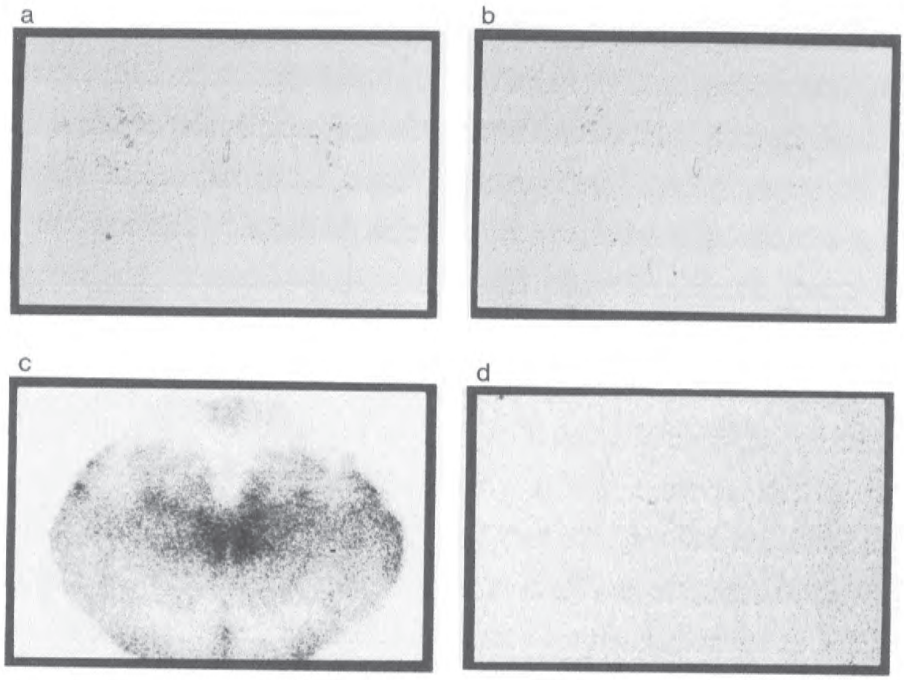

Figure 6.6: Spinal cord sections lightly stained with cresyl violet (a, b) and autoradiographs showing the binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to adjacent fresh (unfixed) spinal cord sections in the absence (c) or presence (d) of 10 μM RαCGRP.

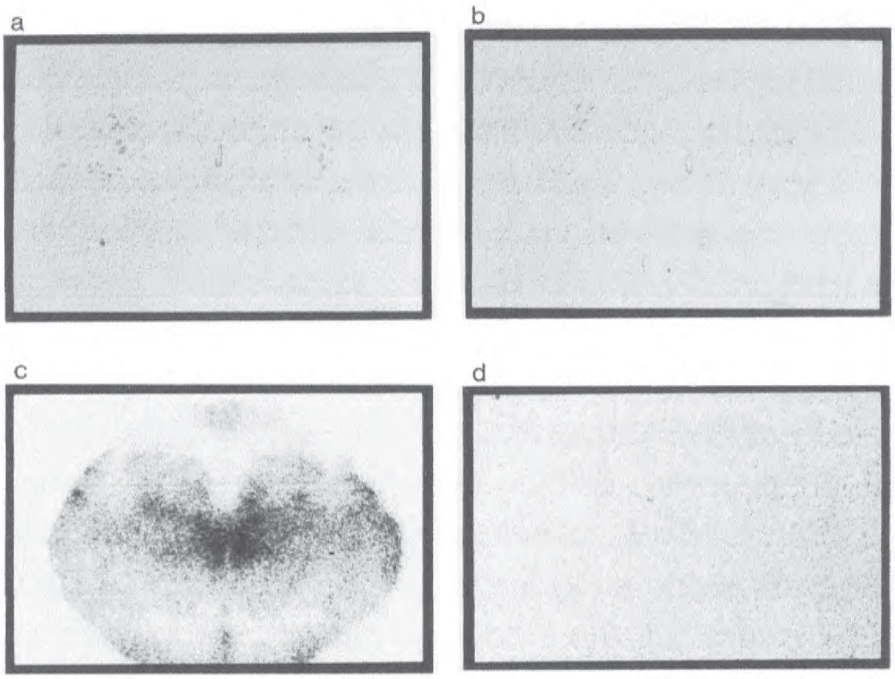

Figure 6.6: Spinal cord sections lightly stained with cresyl violet (a, b) and autoradiographs showing the binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to adjacent fresh (unfixed) spinal cord sections in the absence (c) or presence (d) of 10 μM RαCGRP.

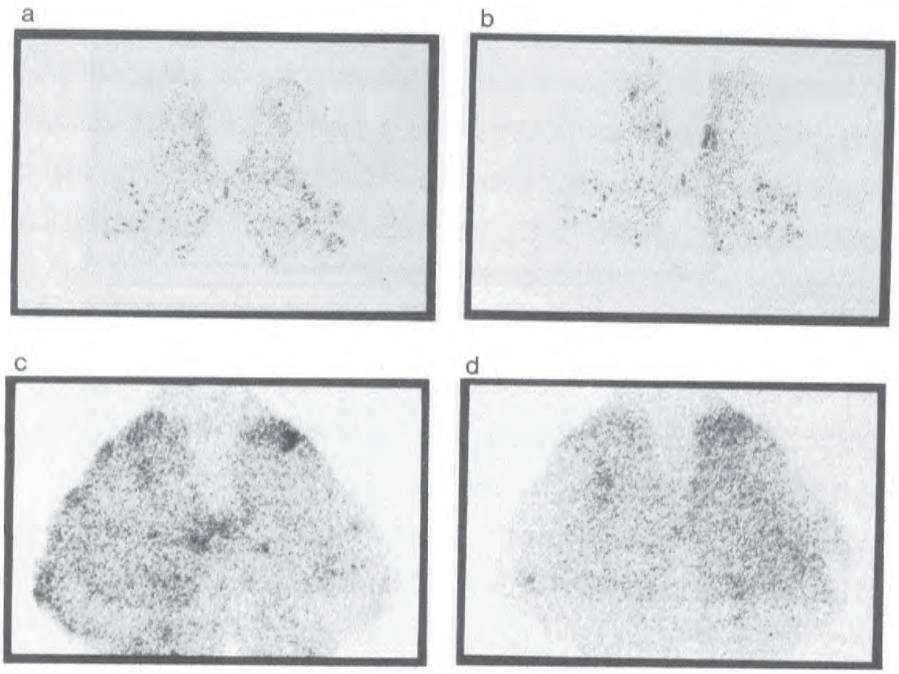

Figure 6.7: Cresyl violet stained spinal cord sections showing tissue morphology (a, b) and autoradiographs showing the binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to adjacent paraformaldehyde-fixed spinal cord sections in the absence (c) or presence (d) of 10 μM RαCGRP.

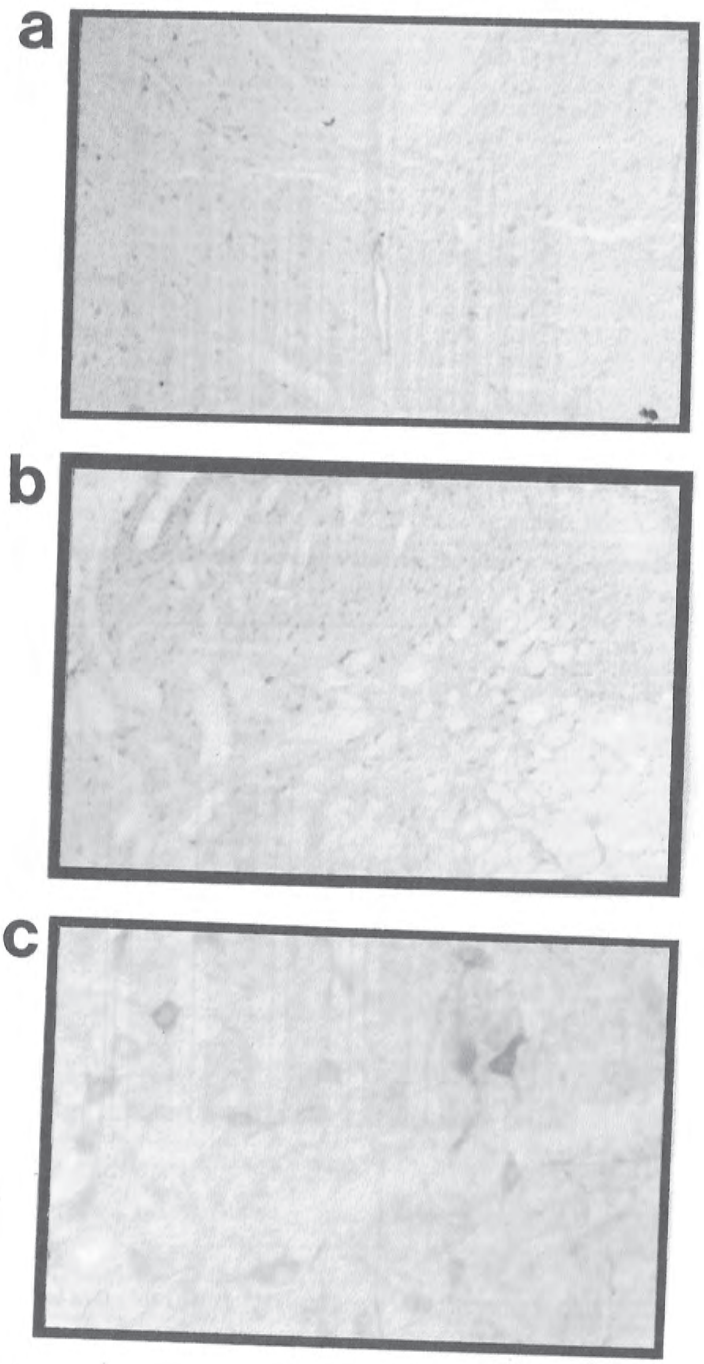

Figure 6.8: Immunostaining of fresh spinal cord sections with MAb Id1 at 15 μg/ml (a; central canal; x100), MAb Id3 at 30 μg/ml (b; dorsal horn; x100), and MAb Id12 at 60 μg/ml (c; dorsal horn; x400).

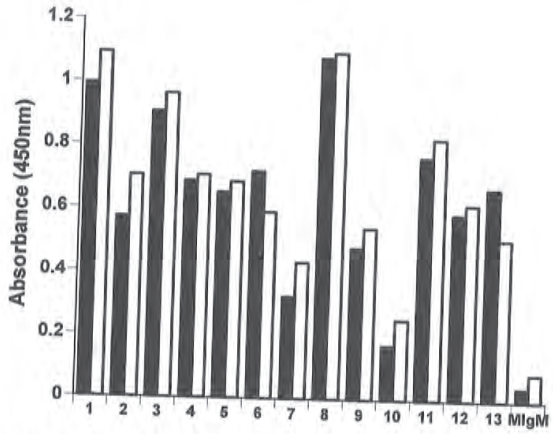

Figure 6.9: Effect of co-incubation with vehicle (filled bars) or RαCGRP (open bars) on the binding of Id MAbs (1 to 13) to rabbit affinity-purified anti-CGRP PAbs by indirect ELISA. Non-specific mouse IgM (10 μg/ml; MIgM) was used as a control. Values are the mean of two observations.

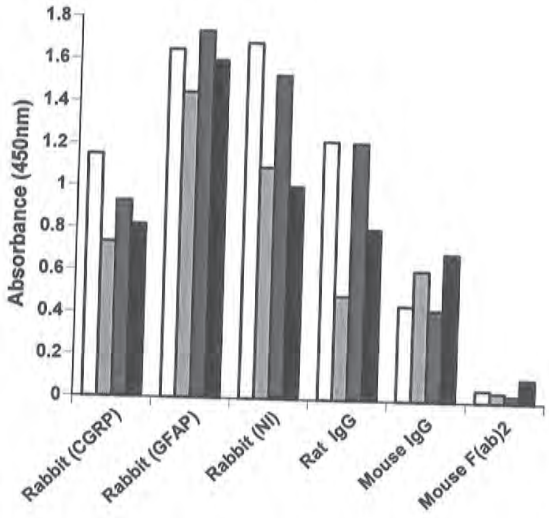

Figure 6.10: Binding of MAbs Id1 (white), Id2 (light grey), Id3 (dark grey) and Id4 (black) to rabbit anti-CGRP, anti-glial fibrillary acidic protein (GFAP), and non-immune (NI) IgG, normal rat IgG, mouse MAb R1.50 IgG and MAb R1.50 $F(ab')_2$ fragments by indirect ELISA. Similar binding was observed for MAbs Id 5 to 13. Values are the mean of two observations.

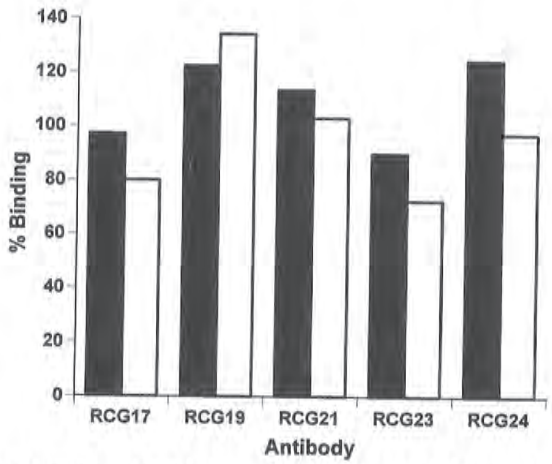

Figure 6.11: Effect of RCG MAbs on the binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to human neuroblastoma SK-N-MC cell (filled bars) or rat brain (open bars) membrane preparations. Binding is expressed as a percentage of specific binding in the presence of cell culture medium (10FD) alone. The final dilution of hybridoma supernatants and blank medium was 1 in 5 in the binding assays. Each value is the mean of two observations.

6.4. Discussion

The present investigations have systematically addressed the major questions of whether Id MAbs raised by an auto-anti-idiotypic approach and RCG MAbs raised against a CGRP receptor purified from porcine cerebellum specifically bind to CGRP receptors in rat tissues and in rat and human cell lines.

Id MAbs were screened primarily by receptor binding assays and immunocytochemistry. The series of questions posed during the present study and the experiments designed to answer them are summarized in Table 6.1. The competition of internal image anti-idiotype with antigen should be demonstrable with cell types for different species and not restricted to a particular idiotype-anti-idiotype interaction (Bona & Kohler, 1984). Binding studies have therefore been performed in rat tissue membranes as well as membranes from the human SK-N-MC cell line. Id MAbs clearly did not inhibit CGRP binding to 3 different membrane preparations. It could be argued that the primary screen to detect antibodies with the desired internal image properties should be an assessment of whether the interaction between Id MAbs and the surrogate receptor (anti-CGRP antibodies) could be inhibited by the ligand (CGRP). However, inhibition of CGRP receptor binding by potential anti-receptor antibodies was the primary criterion of success in this study. Indeed, the ability of an

Table 6.1: Summary of questions posed in chronological order during the present study of Id MAbs and experiments designed to answer the questions.

Question	Experiment
Can Id MAbs displace the binding of CGRP to its receptor?	Receptor binding assay
Do Id MAbs bind specifically to a tissue known to have high density of CGRP receptors?	Immunocytochemistry (rat spinal cord)
Is the distribution of specific binding by immunocytochemistry similar to autoradiographic localization of CGRP receptors?	Receptor autoradiography
Is specific binding by immunocytochemistry displaceable by CGRP?	Immunocytochemistry
Does paraformaldehyde fixation of tissues denature CGRP receptors?	Receptor autoradiography
Can Id MAbs bind to cell lines with CGRP receptors compared with cell lines that do not express CGRP receptors?	Immunocytochemistry (rat and human cells)
Is the binding of Id MAbs to affinity-purified anti-CGRP PAbs displaceable by CGRP?	ELISA
Do the Id MAbs bind non-specifically to rabbit immunoglobulins?	ELISA
Are Id MAbs anti-Fc fragment antibodies which bind immunoglobulins of different species?	ELISA

anti-idiotypic antibody to act like a natural ligand for a cellular receptor has been stated as a primary functional criterion of an internal image Ab2β.

Immunocytochemistry was used as a screening procedure because it may be a more sensitive technique. Compared with receptor binding assays, there was no need, at least initially, to inhibit binding in order to detect a positive result. This could facilitate the detection of lower affinity antibodies and, of lesser interest, antibodies not directed against the ligand binding site. In addition, receptor localization by immunocytochemistry was of interest as a research technique. The advantages of

immunocytochemical localization of receptors over receptor autoradiography include higher resolution, suitable application at electron microscopic level and cellular localization with the possibility of immunostaining single cells. Unlike autoradiography, the technique does not require the use of radioactivity and results may be obtained rapidly.

CGRP receptors are detectable in the spinal cord by receptor binding assay (Wimalawansa, 1992) and by receptor autoradiography (Yashpal *et al.*, 1992). However, attempts to localize CGRP receptors by immunocytochemistry presented some methodological challenges. The cellular immunostaining observed in spinal cord sections were encouragingly similar to receptors localized by other anti-idiotypic antibodies in the central nervous system (Knigge *et al.*, 1989) and the density of immunostaining was similar to the autoradiographic distribution of CGRP binding sites. The immunostaining was diminished by pre-incubation of MAbs with excess affinity-purified rabbit anti-CGRP PAbs. Thus it was likely that the antigens recognized by the Id MAbs in the rat spinal cord bore the same epitopes as the rabbit anti-CGRP PAbs. The key to the confirmation of specific receptor localization was CGRP-displaceable binding but the autoradiography experiments showed that fixation denatured CGRP receptors. Denaturation of receptors by fixatives in immunocytochemistry is a problem that has been encountered by others (Wang *et al.*, 1990). Salih *et al.* (1979) studied the effect of a range of fixatives on the stability of prolactin receptors and found that, depending on the fixative used, the binding capacity of receptors could be significantly reduced or completely destroyed. Non-specific binding was higher following some fixation procedures, as observed in the present study with paraformaldehyde. In addition, there were tissue and species differences in the susceptibility of receptors to denaturation by fixatives.

Fixation is critical in many immunocytochemical procedures. The primary purpose of fixation is to preserve the relevant antigen and the morphology of cells. Failure to fix a soluble antigen would result in its disappearance through washing. However, it was hypothesized that fixation might not be necessary for the localization of a membrane-bound receptor. Good tissue morphology and immunostaining was observed with unfixed, slide mounted, tissue sections. However, CGRP displacement experiments were not performed because of the implications of the ELISA experiments. The advantages of immunostaining live and fixed cells were not realized because none of the Id MAbs showed positive staining of SK-N-MC and L6 cells known to express CGRP receptors.

An anti-idiotypic antibody may recognize an epitope on a receptor that is not the ligand binding site (for example, see Cayanis *et al.*, 1986). Western blotting of the CGRP receptor with Id MAbs would be a method for screening for MAbs that do not

recognize the ligand binding site. However, the desired use of these MAbs was as CGRP antagonists, and recognition of the ligand binding site was considered an important criterion for antibodies to function as receptor antagonists. Moreover, screening by immunoblotting requires the MAbs to recognize denatured receptor.

The difficulties in demonstrating specific receptor binding led to the fundamental question of whether the Id MAbs had internal image properties which could be demonstrated by CGRP displacement in an ELISA. An irrelevant affinity-purified rabbit PAb preparation against glial fibrillary acidic protein was included as a control. All the Id MAbs were found to be strongly positive against the rabbit PAbs. This was the first indication that the Id MAbs were anti-immunoglobulin antibodies. The finding was readily confirmed with other immunoglobulins from rabbit, rat and mouse. Id MAbs were most probably anti-Fc fragment antibodies since they bound MAb R1.50 IgG but not $F(ab')_2$ fragments by ELISA. The lack of binding of Id MAbs to the $F(ab')_2$ fragment of MAb R1.50, an anti-CGRP antibody that inhibits the binding of CGRP to its receptor, is also additional evidence that the Id MAbs do not possess internal image properties. The positive immunostaining detected in rat spinal cord sections was probably due to localization of membrane-associated immunoglobulins through binding to Fc fragments.

The use of affinity-purified rabbit anti-ligand PAbs as a surrogate receptors for the screening of auto-anti-idiotypic antibodies was advocated by Cleveland & Erlanger (1986). However, the use of affinity-purified rabbit anti-CGRP PAbs alone for the screening of hybridomas was insufficient. Inclusion of purified non-immune rabbit immunoglobulins as a control in the ELISA would have eliminated the false positives. It was assumed that this had been done throughout the collaboration. However, the non-specific anti-immunoglobulin characteristics were only discovered following cloning of the Id MAbs and after much effort into screening for receptor binding properties. Screening with Fab fragments of anti-CGRP PAbs would also reduce the possibility of false positives due to antibodies directed against Fc fragments such as rheumatoid factors that arise after every type of immunization (Carson *et al.*, 1987)

It may be concluded that the Id MAbs are not Ab2β internal image anti-idiotypic antibodies. However, they may still be a particular class of anti-idiotypic antibody. Bona *et al.* (1982) have described anti-idiotypic antibodies that specifically recognize idiotopes on Fab' fragments of immunogen antibodies (Ab1) that also bind to Fc fragments. Such anti-idiotypic antibodies which bind to a framework idiotope and to an epitope on the antigen have been termed Ab2ε (Bona & Kohler, 1984). It has been postulated that this class of anti-idiotypic antibody is restricted to some rheumatoid factor which possesses a paratope (combining site) with high binding

affinity for a given idiotope and which also exhibits a low affinity for Fc fragment. Rheumatoid factors have been hypothesized to be anti-idiotypic antibodies against virus-induced anti-Fc receptor antibodies (Mouritsen, 1986).

The RCG MAbs were raised against a receptor purified from porcine cerebellum (Wimalawansa *et al.*, 1993). MAb RCG23, for example, has been shown to inhibit CGRP binding to solubilized and membrane bound receptors from this tissue by more than 50% at a supernatant dilution of 1 in 1000 (Wimalawansa, 1992). However, the RCG MAbs did not inhibit CGRP binding to rat and human membrane preparations at a low dilution (1 in 5) of the hybridoma supernatants. Lack of immunostaining of CGRP receptors in rat brain sections by RCG MAbs has been found by others (A. Herbison, personal communication). The most like explanation for the discrepancies in experimental results is failure of these anti-porcine CGRP receptor MAbs to recognize the rat CGRP receptor. Experience in raising MAbs against the insulin receptor has revealed considerable species divergence in receptor structure even in regions close to the insulin binding site (Soos *et al.*, 1986). More recently, it was shown that a single amino acid difference can cause a dramatic pharmacological variation between species homologues of the same receptor (Oksenberg *et al.*, 1992).

The findings with RCG MAbs highlight a shortcoming of the strategy of immunization with purified receptor. The CGRP receptor was purified from porcine rather than rat tissue because of its particular abundance in porcine cerebellum and the availability of large amounts of tissue from the pig (S. Wimalawansa, personal communication; Wimalawansa & El-Kholy, 1993). However, the problem of species variation meant that further experiments could not be done in the most commonly used experimental animal.

In summary, Id MAbs were found not to bind to CGRP receptors because they were not internal image Ab2β anti-idiotypic antibodies. RCG MAbs were found not to bind CGRP receptors in rat and human cells probably because of species differences in receptor structure.

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CHAPTER 7

Pharmacological characterization of immunoblockade by an anti-CGRP monoclonal antibody

7.1. Introduction

Following successful production and biochemical characterization of MAbs against CGRP (Chapters 3 and 4), pharmacological characterization was the next step towards the development of immunoblockade as an experimental technique for probing the physiological role of CGRP. The isolated porcine coronary artery was chosen as the preparation to test whether MAb R1.50 could block the vasodilatory effect of CGRP. An *in vitro* functional test system was chosen for initial studies because this allowed the assessment of immunoblockade independent of pharmacokinetic factors which must be considered *in vivo*.

The dilatation of coronary arteries by CGRP has been well characterized in man (McEwan *et al.*, 1986; Franco-Cereceda, 1991a; Ludman *et al.*, 1991) and in several animal species including the rat (Holman *et al.*, 1986; Prieto *et al.*, 1991) and the pig (Franco-Cereceda *et al.*, 1987; Ezra *et al.*, 1987; Franco-Cereceda, 1991b). CGRP-like immunoreactive fibres are present in the adventitia of human and porcine coronary arteries (Gulbenkian *et al.*, 1993; Shoji *et al.*, 1987). Exposure of the isolated human coronary artery to capsaicin causes an increase in CGRP immunoreactivity outflow (Franco-Cereceda, 1991a). A high number of CGRP receptors have been detected autoradiographically in the main coronary arteries (Coupe *et al.*., 1990; Sun *et al.*, 1993).

Capsaicin produces a relaxation of pre-contracted coronary artery rings and segments. Several lines of evidence suggest that CGRP, rather than substance P co-localized in perivascular sensory fibres, mediates the effect of capsaicin. Vasodilatation by substance P, but not CGRP or capsaicin, is endothelium-dependent. CGRP and capsaicin remain active following induction of tachyphylaxis to substance P (Franco-Cereceda *et al.*, 1987). The responses to capsaicin and CGRP are not affected by gossypol and methylene blue, two inhibitors of endothelium-derived relaxing factor-mechanisms, which block the effect of substance P (Franco-Cereceda & Rudehill, 1989; Franco-Cereceda, 1991a). Capsaicin-induced vasodilatation and release of CGRP is inhibited by ruthenium red, an inhibitor of calcium fluxes (Franco-Cereceda, 1991a).

The co-release of at least two vasodilatory peptides from perivascular nerves by capsaicin presents potential difficulties in investigations of the role of CGRP as an endogenous vasodilator. The isolated porcine coronary artery preparation appeared to

be a "clean" model for such investigations for the reasons mentioned above. Removal of the endothelium allows assessment of the role of endogenous CGRP independently of substance P.

The major objective of the work described in this chapter was to characterize the immunoblocking properties of MAb R1.50 which appeared to be the best candidate as a pharmacological tool from biochemical screening (Chapter 4). Following demonstration of the immunoblockade of exogenous CGRP, attempts were made to block the effect of capsaicin which was postulated to be mediated by CGRP released from perivascular sensory fibres. Whole IgG and Fab' fragments were tested because it was uncertain whether IgG could diffuse into and achieve effective concentrations in the synaptic cleft for immunoblockade. The results of immunoblockade were compared with those obtained by the use of HαCGRP$_{8-37}$ as a CGRP receptor antagonist.

7.2. Methods

Materials	**Supplier**
HαCGRP	Peninsula
HβCGRP	Peninsula
HαCGRP$_{8-37}$	Celltech
Potassium chloride	Sigma
Sodium nitroprusside	Sigma
Capsaicin	Sigma
Substance P	Peninsula
MAb R1.50 IgG	C. Plumpton
MAb R1.50 Fab' fragment	K. Tan
Anti-insulin MAb 22CA2	Department of Clinical Biochemistry, University of Cambridge

Composition of modified Krebs solution (mM; all materials from Sigma)

Na^+ 125, K^+ 5, Ca^{2+} 2.25, Mg^{2+} 0.5, Cl^- 98.5, SO_4^{2-} 0.5, HCO_3^- 32, HPO_4^{2-} 1 and EDTA 0.04; supplemented with Na^+ 15, fumarate 5, pyruvate 5, L-glutamate 5 and glucose 10. Water for the solution was deionized and double glass distilled.

Materials	*Stock (g/L)*	*Addition (ml) to final 10 L solution*
NaCl	104.2	500
$NaHCO_3$	48.8	500
KCl	18.6	200

$Na_2HPO_4.12H_2O$	17.9	200
$MgSO_4.7H_2O$	6.0	200
$EDTA,Na_2H_2O$	14.98	10
$CaCl_2$	(1 M)	22.5

The solution was gassed with 95% O_2:5% CO_2 before addition of $CaCl_2$

"Supplement"	*Stock solution (g/l)*
$NaHCO_3$	63.0
Fumaric acid	29.0
Glutamic acid	36.75
Glucose	90.5
Sodium pyruvate	27.5

0.5 ml of "supplement" stock solution was added to 24.5 ml of Krebs solution in tissue bath.

Capsaicin was initially dissolved in 70% ethanol and further diluted in modified Krebs to the appropriate concentration for addition to tissue baths to give a final concentration of 10^{-5} M or 10^{-6} M. MAb R1.50 was in the form of ascites fluid purified by ammonium sulphate precipitation (Chapter 4). The antibody vehicle was PBS containing 0.05% thiomersal as a preservative. All other drugs were made up in modified Krebs.

7.2.1. Experimental procedures

Fresh pig hearts were collected from the local abattoir and transported to the laboratory in ice. Epicardial coronary artery ring segments (2-3 mm, 0.5-1 mm in diameter) were dissected, denuded of endothelium by light rubbing, and mounted in 25 ml tissue baths containing Krebs solution, maintained at 37°C, and aerated with 95% O_2:5% CO_2. The artery segments were attached to strain-gauge transducers (Swema SG4-45) which were connected to HSE 2-channel bridge amplifiers and a chart recorder (Watanabe 8 channel linear corder Mark VII) for the recording of isometric tension. A resting tension of 5 mN (0.5 g weight) was applied. The artery segments were pre-contracted with 25 mM potassium chloride (KCl). Two stable and reproducible contractions with KCl were obtained before the effects of drugs were tested. Effective removal of endothelium from arterial segments was initially confirmed by lack of response to 100 nM substance P. It was found that capsaicin and CGRP could induce relaxation of vessels following endothelium removal and tachyphylaxis to substance P, as reported by Franco-Cereceda *et al.* (1987). Sodium nitroprusside (10^{-4} M) was added at the end of experiments to confirm that a maximal relaxant response could be elicited from each artery segment. Only vessels that demonstrated maximal relaxation to sodium nitroprusside were included in the data

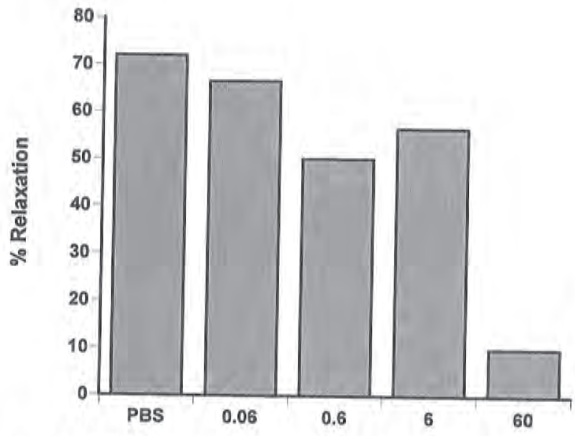

Figure 7.1: Effect of MAb R1.50 concentration on the relaxation of porcine coronary artery segments by 50 nM HβCGRP. Coronary artery segments were pre-contracted with 25 mM KCl and pre-incubated with MAb R1.50 for 60 minutes prior to exposure to HβCGRP. Control tissues were pre-incubated with PBS vehicle for 60 minutes. Values are the mean of 2 observations.

analysis. Each experiment was performed on tissues from at least 3 animals.

7.2.2. Blockade of responses to exogenous CGRP

The effective blocking concentration of MAb R1.50 was found by studying the effect of MAb concentration on the response to 50 nM HβCGRP which produces a maximal relaxation of coronary artery segments. Tissues were incubated with MAb R1.50 IgG for 60 minutes prior to exposure to CGRP. HαCGRP or HβCGRP were added to the tissue baths in cumulative fashion in concentration-response studies. A purified anti-insulin MAb (coded 22CA2) was used as a control MAb IgG.

The effect of MAb R1.50 Fab' fragments on responses to HαCGRP was also investigated. The concentration of Fab' fragments used (60 μg/ml) was chosen on the basis of similar CGRP binding characteristics compared with the same concentration of IgG by ELISA (Chapter 4). MAb R1.50 Fab' fragment was added to tissue baths 60 minutes prior to the start of CGRP concentration-response studies.

The effect of $HαCGRP_{8-37}$ as an antagonist of HαCGRP was tested at concentrations of 1 μM and 5 μM. $HαCGRP_{8-37}$ was added to tissue baths 30 minutes prior to the start of CGRP concentration-response studies.

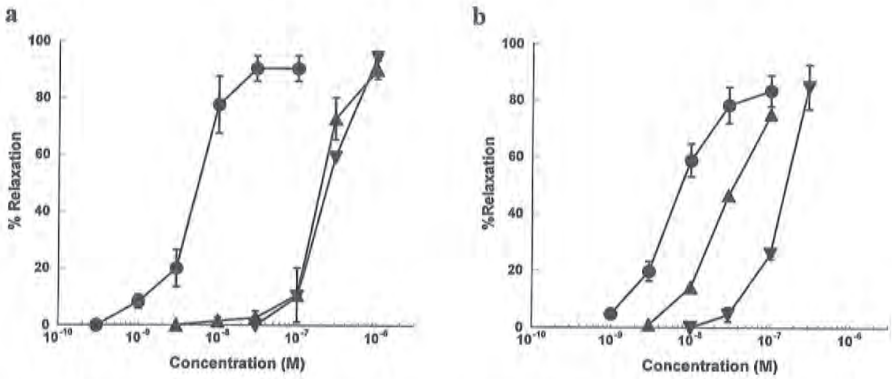

Figure 7.2: Effect of MAb R1.50 IgG at 12 μg/ml (triangles) or 60 μg/ml (inverted triangles) on the relaxation of KCl pre-contracted coronary artery segments by HαCGRP (a) and HβCGRP (b). Control concentration-response relationships were obtained in the presence of PBS vehicle. Mean values and standard error bars are shown where there were 4 to 6 observations. Where no error bars are shown, values are the mean of 2 observations.

7.2.3. Blockade of responses to capsaicin

Concentrations of MAb R1.50 IgG (60 μg/ml), MAb R1.50 Fab' fragments (60 μg/ml) and $H\alpha CGRP_{8-37}$ (5 μM) which blocked exogenous CGRP effectively were used in attempts to block capsaicin-induced relaxation of pre-contracted artery segments. Preliminary studies showed that 10 μM capsaicin was required to induced a significant relaxation (about 20%). Tissues were incubated with MAb R1.50 IgG and Fab' fragments for 3 hours prior to exposure to 10 μM capsaicin whilst $H\alpha CGRP_{8-37}$ was added 30 minutes before 10 μM capsaicin.

7.2.4. Data analysis

The relaxation of pre-contracted coronary artery segments was expressed as a percentage of stable pre-dose contractions. The *t* test for unpaired data was used for statistical comparisons. A *P* value of less than 0.05 was considered statistically significant.

7.3. Results

7.3.1. Blockade of responses to exogenous CGRP

The concentration-ranging experiment showed that a 60 minute pre-incubation of tissues with 60 μg/ml of MAb R1.50 IgG markedly inhibited the relaxant effect of 50 nM CGRP (Figure 7.1). CGRP caused concentration-dependent relaxations of pre-contracted artery segments. Similar concentration-response curves were obtained for the two forms of CGRP (Figure 7.2). Two concentrations of MAb R1.50 IgG (12 or 60 μg/ml) were found to produce apparent parallel shifts in the concentration-response curve of CGRP. A difference in the rightward shift due to the two concentrations of MAb R1.50 was evident for HβCGRP but not for HαCGRP (Figure 7.2). The response to CGRP concentrations up to 30 nM was completely abolished by pre-incubation with 60 μg/ml MAb R1.50 IgG (Figure 7.3). Anti-insulin MAb 22CA2 at 60 μg/ml (n=2) did not affect the concentration-response relationship of CGRP.

Like the whole IgG, MAb R1.50 Fab' fragment at 60 μg/ml shifted the CGRP concentration-response relationship to the right (Figure 7.4). $H\alpha CGRP_{8-37}$ produced rightward parallel shifts of the CGRP concentration-response relationship in a dose-dependent manner (Figure 7.5). The rightward shift of the CGRP concentration-response relationship due to 5 μM $H\alpha CGRP_{8-37}$ or 60 μg/ml MAb R1.50 Fab' fragment were very similar .

7.3.2. Blockade of responses to capsaicin

Pre-incubation of tissues with MAb R1.50 IgG for 3 hours at a concentration which clearly shifted the CGRP concentration-response curve rightward and which markedly inhibited the maximal response to CGRP produced no significant effect on the relaxant response to capsaicin (Figure 7.6).

A lower response to capsaicin was observed in the presence of MAb R1.50 Fab' fragment compared with vehicle control but the difference was not statistically significant (Figure 7.6). In contrast, $H\alpha CGRP_{8-37}$ appeared to have augmented the response to capsaicin but, again, the effect was not statistically significant (Figure 7.6).

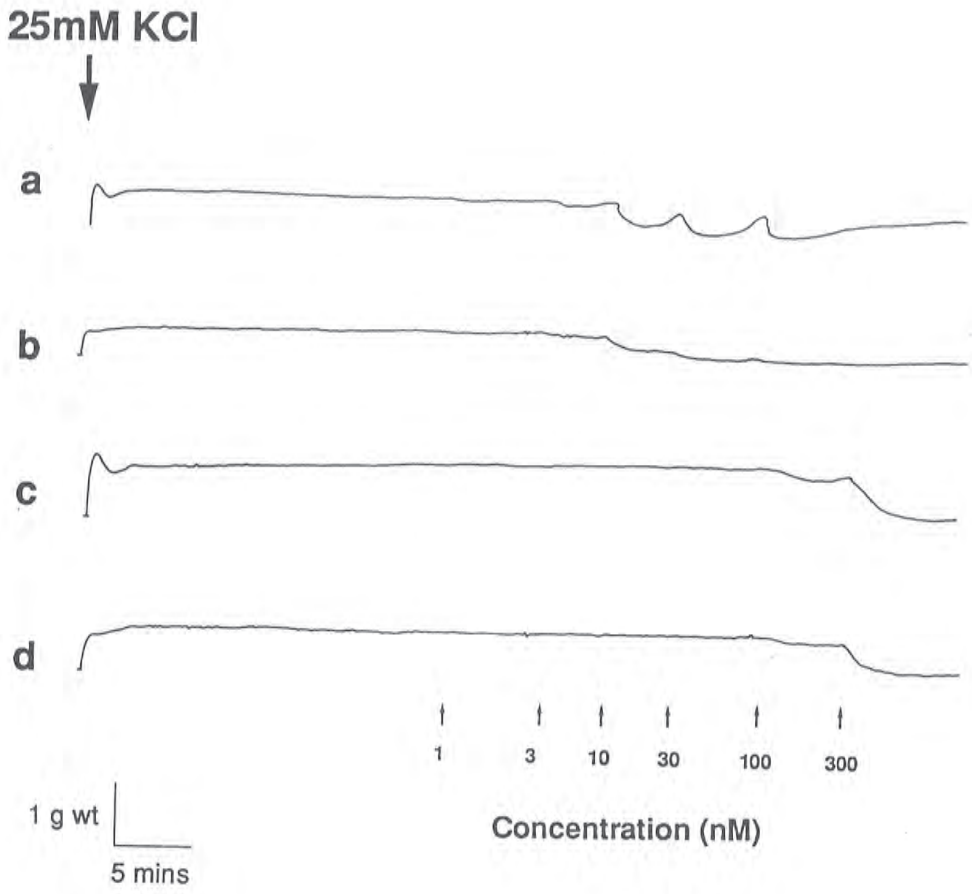

Figure 7.3: Original traces showing the relaxation of KCl pre-contracted coronary artery segments by HβCGRP in the presence of PBS vehicle (a,b) or 60 μg/ml MAb R1.50 IgG (c,d). MAb R1.50 or vehicle was added to tissue baths 60 minutes prior to the start of the CGRP concentration-response study.

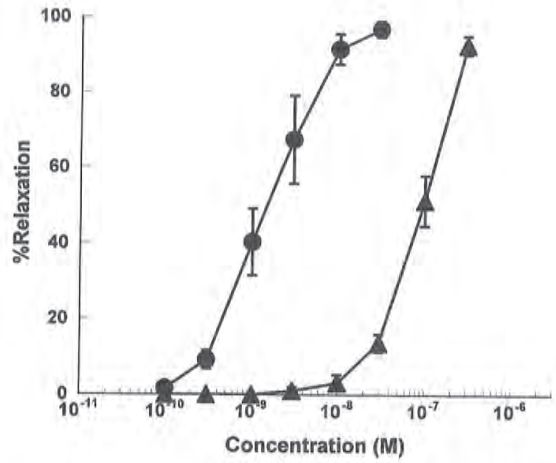

Figure 7.4: Effect of MAb R1.50 Fab' fragments (triangles) or PBS vehicle (circles) on the relaxation of KCl pre-contracted porcine coronary artery segments by HαCGRP. Tissues were incubated with the Fab' fragment or PBS vehicle for 30 minutes before the start of the CGRP concentration-response study. Mean and standard error bars are shown (n=6).

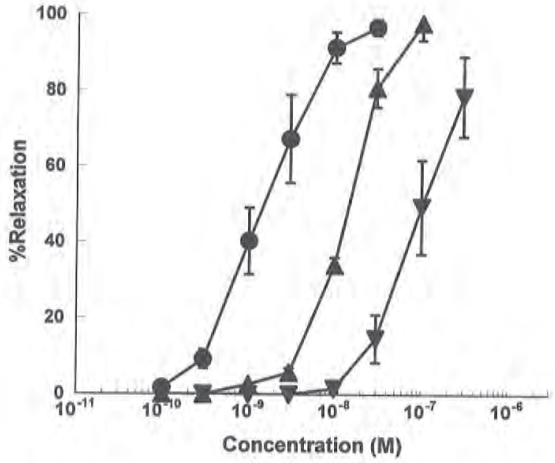

Figure 7.5: Effect of HαCGRP$_{8-37}$ at 1 μM (triangles) or 5 μM (inverted triangles) on the relaxation of KCl pre-contracted porcine coronary artery segments by HαCGRP compared with control (PBS; circles). HαCGRP$_{8-37}$ or PBS was added to tissue baths 60 minutes before the start of the CGRP concentration-response study. Mean and standard error bars are shown (n=5 to 6).

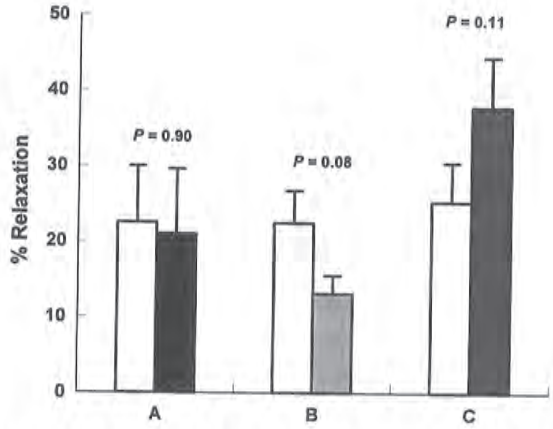

Figure 7.6: Effect of 60 μg/ml MAb R1.50 IgG (A; black bar), 60 μg/ml MAb R1.50 Fab' fragment (B; light grey bars) or 5 μM HαCGRP$_{8-37}$ (C; dark grey bar) on capsaicin-induced relaxation of KCl pre-contracted coronary artery segments compared with concurrent vehicle controls (A, B, and C; white bars). A single concentration of capsaicin (10 μM) was tested 3 hours after incubation with MAb R1.50 IgG or Fab' fragments. Capsaicin was added to tissue baths 30 minutes after H αCGRP$_{8-37}$. Mean values and standard error bars are shown (n=6 to 12).

7.4. Discussion

The ability of the MAb R1.50 to block CGRP added to the tissue bath was clearly established in this study. However, the role of endogenous CGRP in mediating the effect of capsaicin could not be defined in the present investigations because neither MAb R1.50 nor HαCGRP$_{8-37}$ blocked the response to capsaicin significantly.

The binding of an antibody to a biologically active peptide does not always lead to the blockade of its effects. The need to characterize the immunoblocking properties of a MAb prior to further application is emphasized by the findings of Shaw *et al.* (1992) that anti-CGRP MAbs directed against different epitopes could block, augment or have no effect on CGRP-induced vasodilatation in porcine coronary artery rings. The present experiments with exogenous CGRP served as positive controls for the immunoblockade of CGRP that may be released from perivascular nerves.

The response to capsaicin was not blocked by MAb R1.50 IgG at a concentration which clearly shifted the CGRP concentration-response curve rightward and which markedly inhibited the maximal response to CGRP. There are several possible explanations for the negative observation. First, the MAb might not have

recognized porcine CGRP. Second, the relatively large IgG immunoglobulin molecule (molecular weight 150 kDa) might not have diffused into the space between nerve and vascular tissues. Third, other vasodilatory neuropeptides could have been released by capsaicin. Fourth, capsaicin may have a direct, non-specific, relaxant effect on vascular smooth muscle. Last, any difference may be difficult to detect due to between-tissue variability in responses to capsaicin.

R1.50 is a non-selective MAb against the rat and human forms of CGRP which differ in N- and C-terminal sequences. Although the sequence of porcine CGRP differs from that of HαCGRP by 6 amino acids, there is only a three amino acid difference from RαCGRP at positions 22, 25 and 31 (see Figure 1.1; Chapter 1). The amino acids at positions 22 and 25 are identical for porcine CGRP and HβCGRP. It is unlikely that R1.50 would not recognize porcine CGRP. However, the non-availability of synthetic porcine CGRP meant that immunoblockade experiments could not be performed. Recognition of porcine CGRP by MAb R1.50 could have been investigated by immunocytochemistry using porcine tissues but this would not provide useful information concerning the immunoblocking properties of the antibody. MAb R1.50 was subsequently found to be a poor blocker of RαCGRP (see Chapter 8) despite binding to this species of CGRP by ELISA and immunocytochemistry.

At the time of the study, it was unclear if whole IgG molecules could diffuse into the synaptic cleft for *in vitro* immunoblockade of endogenous CGRP within the experimental time-scale. It was anticipated that an even greater problem with distribution limitations would be encountered *in vivo*. Fragmentation of MAb R1.50 had been performed in order to investigate this (Chapter 4). The negative results with the whole IgG prompted testing of the Fab' fragment. The findings with the Fab' fragment was also equivocal. The response to capsaicin was lower in presence of the Fab' fragment but the result was not statistically significant.

The antagonistic activity of $H\alpha CGRP_{8-37}$ was reported in the course of the present investigations. This afforded an opportunity to examine a more conventional approach to the problem. $H\alpha CGRP_{8-37}$ blocked the effect of exogenous CGRP but did not affect the response to capsaicin significantly. The present observations are not in agreement with the finding of Franco-Cereceda (1991b) that $H\alpha CGRP_{8-37}$ completely abolished the relaxant response to 10 μM capsaicin in porcine coronary artery rings.

Vasodilatory neuropeptides other than CGRP and substance P, such as vasoactive intestinal peptide, could be released by capsaicin from perivascular fibres (Gulbenkian *et al.*, 1993). However, this is unlikely if the response to capsaicin could be abolished by $H\alpha CGRP_{8-37}$ (Franco-Cereceda, 1991b) A direct, non-specific,

effect of capsaicin is a possible explanation of the lack of effect of MAb R1.50 and HαCGRP$_{8-37}$ in the present study. Such non-specific effects on smooth muscle contractility are well documented at capsaicin concentrations above 10 μM (Maggi & Meli, 1988). The specific effect of capsaicin on sensory neurons is usually characterized by desensitization. In the present study, a single capsaicin concentration was investigated in each tissue to avoid the development of tachyphylaxis. However, it was subsequently found that repeated doses of capsaicin separated by a 40 minute interval produced a similar relaxant response.

The responses to capsaicin were found to be highly variable between tissues. In the absence of MAb R1.50, the relaxation response to 10 μM capsaicin ranged from 6 to 60%. Variability in coronary artery diameter could be an explanation of this variability. Distal small-diameter coronary artery sections have been shown to be more densely innervated with CGRP-containing nerves (Gulbenkian *et al.,* 1993) and possess a higher number of CGRP binding sites (Sun *et al.,* 1993) than proximal larger-diameter sections. In addition, the potency of CGRP in relaxing porcine coronary artery rings is higher in small-diameter (< 1 mm) than in larger-diameter (3-4 mm) rings (Foulkes *et al.,* 1991). Variable responses to capsaicin were detected in tissues with similar responses to CGRP in the present study. Therefore, differential innervation is a more likely explanation for the variable responses to capsaicin.

The isolated porcine coronary artery proved not to be the ideal *in vitro* preparation for the evaluation of immunoblockade. Porcine CGRP was not available for the critical control experiment involving immunoblockade of exogenous CGRP. The specificity of capsaicin response could not be confirmed with HαCGRP$_{8-37}$ under the experimental conditions used. Difficulties in standardization of small vessel diameter could have led to variable responses. Finally, the experimental set-up with large tissue baths was not suitable for the screening of a larger number of MAbs available in limited quantities.

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CHAPTER 8

Demonstration of the neurotransmitter role of CGRP by immunoblockade with monoclonal antibodies against CGRP

8.1. Introduction

The major objective of the study described in this chapter was to demonstrate the role of CGRP as a neurotransmitter by immunoblockade with anti-CGRP MAbs. The release of endogenous CGRP from primary afferent neurons was achieved through the use of capsaicin. Both capsaicin and CGRP inhibit the nerve-mediated contractions of the isolated rat vas deferens. It was therefore hypothesized that CGRP might be involved in neurotransmission at the neuroeffector junction of the rat vas deferens.

Analysis of the effects of individual endogenous neuropeptides is often difficult because of the co-release of several neuropeptides by capsaicin in many tissues. In particular, tachykinins co-released with CGRP often produce a similar biological response (e.g., dilatation of arteries). In the electrically-stimulated isolated vas deferens, neurokinin A and substance P enhance contractions (Moritoki *et al.*, 1987) in contrast to the inhibitory effect of CGRP (Goto *et al.*, 1987). Tissue concentrations of tachykinins are low in the rat vas deferens, and substance P and neurokinin immunoreactivity are undetectable by immunocytochemistry in this tissue (Saito *et al.*, 1987). In this respect, the rat vas deferens is a particularly useful model for the investigation of CGRP as a major neurotransmitter.

A further objective of the present investigations was to validate the use of immunoblockade as an experimental tool for probing the physiological role of CGRP. Several MAbs were screened for immunoblockade activity *in vitro* and appropriate experimental designs were used to confirm the specificity of blockade. In addition, appropriate models were fitted to the observed data and limited simulations were performed in order to compare observations with theoretical predictions.

8.2. Methods

Materials	Supplier
RαCGRP	Peninsula
RβCGRP	Peninsula
HαCGRP	Peninsula
HβCGRP	Peninsula
Rat β-endorphin	Peninsula

Capsaicin	Sigma
(-)-Isoprenaline hydrochloride	Sigma
Somatostatin	Sigma
Anti-thyroid stimulating hormone MAb (Code MA/732.162)	Serono
Mouse anti-rat MAb 18.5 (ATCC No. TIB 216)	European Collection of Animal Cell Cultures, Porton Down

A 10 mg/ml stock solution of capsaicin was prepared in 70% ethanol and further diluted with Krebs solution for addition to tissue baths. All other drugs were diluted in Krebs solution.

8.2.1. Tissue bath experiments

Approximately 1.5 cm sections of the prostatic portion of the vas deferens were obtained from male Sprague-Dawley rats (220-300 g). Left and right vas deferens from the same animal were used as test and control tissues. The tissues were mounted for isometric tension recordings in 3 ml siliconized (Sigmacote®, Sigma) tissue baths containing Krebs solution maintained at 37°C, pH 7.4, oxygenated with 95% O_2 and 5% CO_2. The Krebs physiological salt solution had the following composition (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, and glucose 11.1. A resting load of 0.5 g weight tension was applied. Isometric tension changes along the longitudinal axis were recorded with a force transducer connected to a Grass Model 7D Polygraph recorder. Platinum wire electrodes were placed at the top and bottom of the tissue bath for electrical field stimulation. The tissues were electrically stimulated using a Multistim System-D330 (Digitimer Limited) stimulator. The following stimulation parameters were used: frequency 0.2 Hz; pulse width 0.5 milliseconds; voltage 60-80V.

Following a 90 minute equilibration period, over which the tissues were electrically stimulated, anti-CGRP MAbs or controls (see below) were added to the tissue baths. Experiments were performed to determine whether pre-incubation with MAb for different periods affected the response to 10 nM RαCGRP (Table 8.1). In all other experiments, tissues were pre-incubated with MAb or control for 10 minutes prior to the determination of cumulative concentration-response relationships to the appropriate agonists (either CGRP, isoprenaline, rat β-endorphin, or somatostatin). Preliminary experiments with CGRP showed that a second concentration-response determination produced a lower maximal effect. Therefore, only one cumulative concentration-response relationship was obtained for CGRP in each tissue. Since tachyphylaxis develops rapidly to somatostatin (Vizi *et al.*, 1984), the response to a single concentration of somatostatin was tested only once in each tissue. Responses to isoprenaline and rat β-endorphin did not desensitize. Cross-over experiments were

Addition of footnotes to Table 8.1 (page 181):

Control response to 10 nM RαCGRP in the absence of MAb C4.19 (Figure 8.4a; page 187) was 72.2% (95% confidence interval 62.6 to 81.9). Control response to 1 μM capsaicin in the absence of MAb C4.19 (Table 8.3; page 194) was 60.8% (95% confidence interval 51.8 to 69.9).

Table 8.1: Effect of incubation period of MAb C4.19 on the mean responses (95% confidence interval) to RαCGRP and capsaicin (% inhibition; tissues from 6 animals at each time point)

Incubation time (minutes)	Response to RαCGRP (10 nM)	Response to capsaicin (1 μM)
10	14.3 (7.6 to 21.0)	55.0 (46.9 to 63.2)*
45	17.8 (9.4 to 26.2)	26.0 (19.0 to 33.1)
90	20.2 (11.8 to 28.6)	29.4 (21.2 to 37.5)

*$p<0.05$ compared with responses at 45 or 90 minutes.

Inhibition of electrically-stimulated contractions of the isolated rat vas deferens was expressed as % inhibition relative to stable contractions recorded immediately before addition of rat αCGRP or capsaicin.

therefore performed in which the same tissue was pre-incubated for 10 minutes with anti-CGRP MAb or control.

Time course experiments were performed to investigate the influence of MAb incubation time on the response to capsaicin (Table 8.1). The effect of MAb C4.19 on the response to capsaicin was compared with controls following a 45 minute incubation. A concentration of capsaicin producing maximal response (1 μM) was used (Maggi *et al.*, 1993). Individual tissues were exposed to a single concentration of capsaicin.

MAbs were added in the form of neat ascites fluid or MAbs purified by ammonium sulphate precipitation of ascites fluid (Chapter 4). Seven MAbs against human CGRP C- and N-terminal fragments (P2.17, P3.15, P3.24, P3.71, P3.77, P5.17 and P5.30 from Dr. C. Plumpton, Clinical Pharmacology Unit) were tested along with the four MAbs (C4.6, C4.19, R1.50, R2.73) obtained from immunization of mice with RαCGRP (Chapters 3 and 4). The MAb vehicle (PBS) was used as a control. A mouse anti-rat MAb MAR 18.5 or a mouse anti-thyroid-stimulating hormone (TSH)

MAb were also used as irrelevant antibody controls at the same protein concentration as anti-CGRP MAbs.

8.2.2. Modelling of immunoblockade

Given the dissociation constant of antibody binding to ligand (Kd), the concentration of binding sites (B_{max}), and the total concentration of ligand (C), the concentration of ligand bound (B(C)) could be predicted from the following equilibrium binding model:

$$B(C) = \frac{B_{max} \times C}{Kd + C}$$

The estimation of Kd and B_{max} from RIA experiments is described in Chapter 4. The concentration of unbound (total - bound) CGRP was simulated for the range of concentrations used in tissue bath experiments. The pharmacological response in the presence of MAb could then be simulated with the pharmacodynamic model described below.

The inhibitory effect of CGRP in tissue bath experiments was expressed as % inhibition relative to stable pre-dosing contractions. The following sigmoidal E_{max} (logistic) model was fitted to concentration-response data obtained from individual tissues:

$$E = E_0 + \frac{E_{max} \times C^N}{EC_{50}^N + C^N}$$

where E is the effect observed in the presence of concentration C, E_0 the baseline effect, E_{max} the maximum effect, EC_{50} the concentration which produces half-maximal effect and N the steepness factor (Hill coefficient).

Parameter estimation and simulation were performed by use of the non-linear extended least-squares regression package MKMODEL version 4.72 (Biosoft, Cambridge).

8.2.3. Statistical analysis

EC_{50} was assumed to be log-normally distributed (Carpenter, 1986). The paired *t* test was performed for comparison of test and control data from paired tissues. One-way

Table 8.2: Estimated geometric mean EC_{50} (95% confidence interval) of drugs following pre-incubation of the electrically-stimulated rat isolated vas deferens with MAb C4.19 or control (nM; n=6 to 8)

Drug	EC_{50} in presence of MAb C4.19 at 60 μg/ml	Control EC_{50} (PBS vehicle[a] or irrelevant MAb MAR 18.5 at 60 μg/ml[b])	Ratio of MAb:Control EC_{50} (95% confidence interval)
RαCGRP	52 (27 to 98)	2.3 (1.5 to 3.6)[a]	21.9 (13.3 to 36.2)
RαCGRP	38 (26 to 54)	4.4 (2.8 to 7.1)[b]	8.5 (7.5 to 9.6)
RβCGRP	32 (21 to 49)	2.4 (1.3 to 4.4)[a]	13.3 (6.2 to 28.4)
Isoprenaline	25 (7.9 to 77)	19 (6.4 to 58)[a]	1.3 (0.3 to 4.8)
Rat β-endorphin	56 (32 to 98)	44 (25 to 76)[a]	1.3 (1.0 to 1.6)

Inhibition of electrically-stimulated contractions of the isolated rat vas deferens was expressed as % inhibition relative to stable contractions recorded immediately before addition of drugs

analysis of variance followed by Tukey's test for multiple comparisons were performed to assess the influence of incubation time on immunoblockade of CGRP and capsaicin responses in unpaired tissues. Ninety-five percent confidence intervals were used to indicate the precision of estimates (Gardner & Altman, 1989). Differences discernible at the 0.05 level were considered statistically significant. Statistical analysis was performed by use of the Statgraphics-Plus package (STSC, Inc., Rockville, U.S.A.).

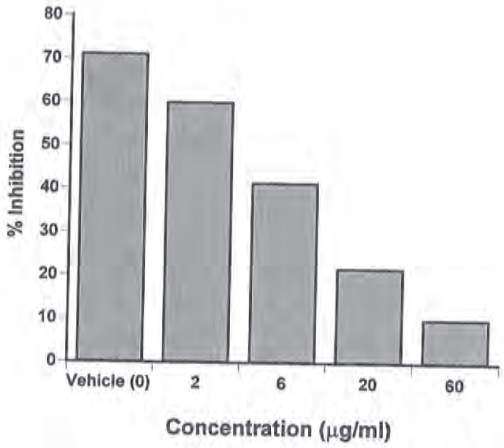

Figure 8.1: Effect of MAb C4.19 at various protein concentrations on the inhibitory effect of 10 nM RαCGRP. The effect of CGRP was expressed as % inhibition relative to stable pre-dosing contractions. Mean responses from 2 experiments are shown.

8.3. Results

8.3.1. Immunoblockade of exogenous CGRP

RαCGRP and RβCGRP produced concentration-dependent inhibition of electrically-stimulated contractions of the isolated rat vas deferens. The two forms of CGRP were equipotent (Table 8.2). Effective blocking concentrations of MAbs were found by assessing the effect of various concentrations of purified MAbs or dilutions of neat ascites fluid on the response to 10 nM RαCGRP. For example, it was found that 60 μg/ml of purified MAb C4.19 ascites was required to block most of the response to 10 nM RαCGRP (Figure 8.1). The effect of a single concentration of MAb on the concentration-response relationship of CGRP was then examined to assess whether an approximate ten-fold $\log_{10}$ shift to the right could be obtained.

None of the MAbs tested had any effect on baseline contractions. Four out of 11 MAbs tested, including MAb C4.19, C4.6 and R2.73 described in Chapters 3 and 4, shifted the concentration-response curve of CGRP to the right compared with vehicle or irrelevant MAb control (Figure 8.2). The use of RIA and a receptor binding assay as biochemical screens (Chapter 4) was generally successful in predicting blocking MAbs. An interesting exception was MAb R1.50 which clearly showed the greatest activity in these assays and in the ELISA (Chapter 4). Although raised in mice immunized with RαCGRP, it was highly potent at blocking HαCGRP but

blocked RαCGRP poorly (Figure 8.3). The unexpected selective blocking activity of MAb R1.50 highlighted the need to perform positive control experiments with exogenous peptide of the form known to be present in the tissue. MAb R2.73 was effective at blocking RαCGRP but not RβCGRP despite cross-reaction with both forms of CGRP in the ELISA.

MAb C4.19 was found to block RαCGRP and RβCGRP effectively and was chosen for detailed studies at a protein concentration of 60 μg/ml (Figure 8.4). Time-course experiments showed that a 10-minute incubation period with MAb C4.19 was sufficient for optimal immunoblockade (Table 8.1). The onset of effect of CGRP was more gradual in tissues incubated with MAb C4.19 than in tissues incubated with PBS vehicle or irrelevant MAb MAR 18.5 (Figure 8.5). The magnitude of the rightward shift (ratio of MAb:control EC_{50}; Table 8.2) of the concentration-response curves for RαCGRP and RβCGRP due to MAb C4.19 were not significantly different. The dissociation constants (Kd) of MAb C4.19 for RαCGRP and RβCGRP were very similar (1.9 and 2.5 nM respectively). Irrelevant mouse MAb MAR 18.5 did not significantly affect the concentration-response relationship of RαCGRP compared with PBS vehicle (Table 8.2). The MAb was purified from ascites fluid by ammonium sulphate precipitation in the same manner as anti-CGRP MAbs. MAb C4.19 had no pharmacologically significant effect on the concentration-response relationship of isoprenaline, rat β-endorphin and somatostatin (Table 8.2, Figures 8.6 to 8.9).

8.3.2. Modelling of immunoblockade

The pharmacological response to CGRP in the presence of MAb C4.19 could be predicted when the dissociation constant and concentration of binding sites of the antibody were known. Comparison of experimental and computer simulated data showed good agreement for EC_{50} and E_{max} of CGRP in the presence of MAb C4.19 (Figure 8.4c). The EC_{50} derived from simulated data was 44 nM. This result was close to the mean EC_{50} of 52 nM (95% confidence interval 27 to 98 nM) estimated from data observed in the presence of MAb C4.19. However, the steepness factor (N) of the theoretical concentration-response curve in the presence of C4.19, 1.81, was significantly higher than the steepness factor of 1.12 (95% confidence interval 0.75 to 1.51) estimated from experimental data.

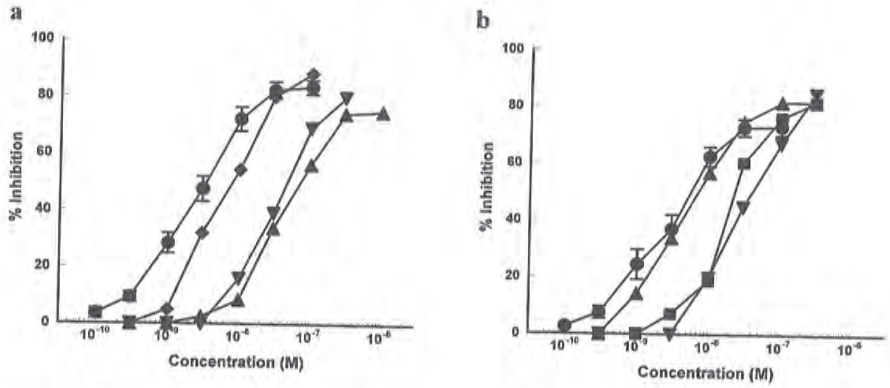

Figure 8.2: Concentration-response curves observed for RαCGRP (a) or RβCGRP (b) in the presence of PBS vehicle (circles) or purified MAbs R2.73 (triangles), C4.6 (inverted triangles), P3.71 (diamonds) and P3.77 (squares) at 60 μg/ml protein. The effect of CGRP was expressed as % inhibition relative to stable pre-dosing contractions. Mean responses to CGRP in the presence of MAbs were data from 2 experiments. Mean responses to CGRP in the presence of PBS are shown with standard error bars (n=8).

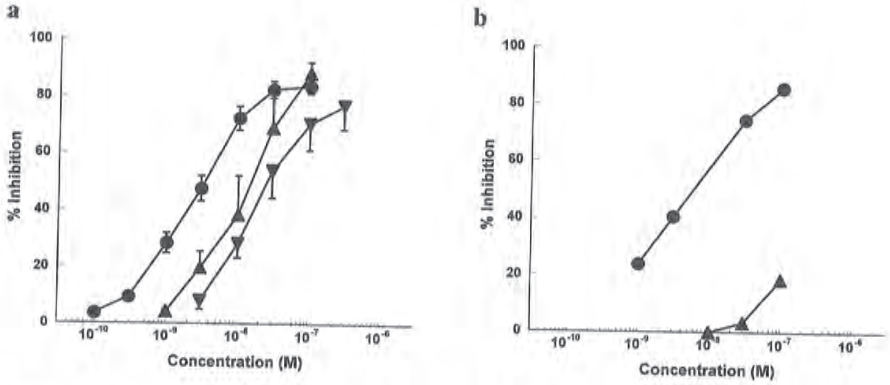

Figure 8.3: Concentration-response curves observed for RαCGRP (a) and HαCGRP (b) in the presence of PBS vehicle (circles) or purified MAb R1.50 at a protein concentration of 60 μg/ml (triangles) or 600 μg/ml (inverted triangle). The effect of CGRP was expressed as % inhibition relative to stable pre-dosing contractions. Mean responses to RαCGRP in the presence of PBS (n=8) or MAb R1.50 (n=3) are shown with standard error bars. Mean responses to HαCGRP in the presence of PBS or MAb R1.50 at 60 μg/ml were data from 2 experiments.

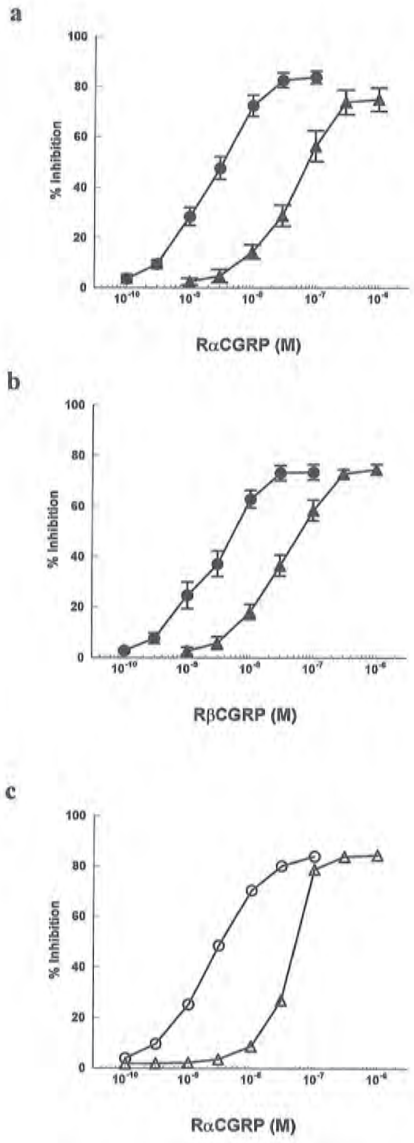

Figure 8.4: Concentration-response curves observed for RαCGRP (a) or RβCGRP (b) in the presence of PBS vehicle (closed circles) or purified MAb C4.19 ascites at 60 μg/ml protein (closed triangles). The effect of CGRP was expressed as % inhibition relative to stable pre-dosing contractions. Mean responses (n=6 to 8) are plotted with standard error bars. Figure 8.4c shows simulated concentration-response curves to RαCGRP in the presence of PBS vehicle control (open circles) or MAb C4.19 (open triangles) at 60 μg/ml protein using estimated Kd of 1.9 nM and B_{max} of 79 nM.

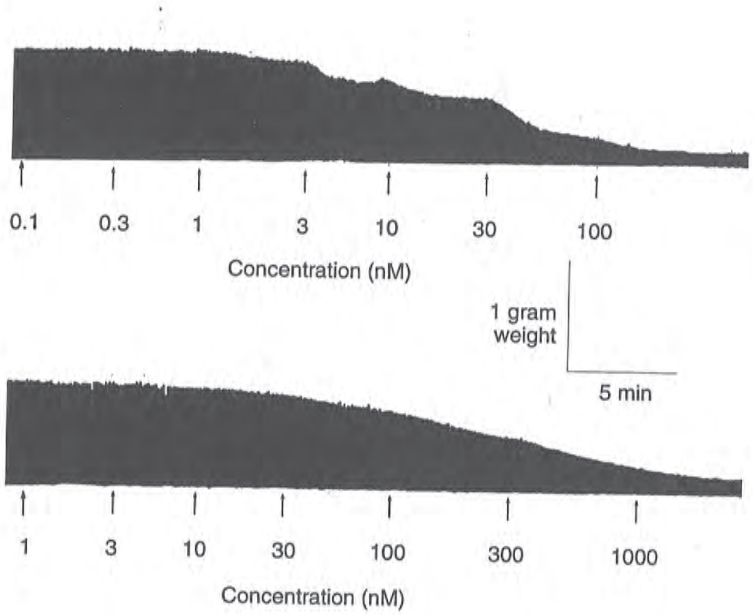

Figure 8.5: Original traces showing the inhibitory effect of RαCGRP on electrically-stimulated contractions of the isolated rat vas deferens in the presence of irrelevant control MAb MAR 18.5 (top) or MAb C4.19 at 60 μg/ml protein (bottom). The traces were obtained from paired tissues from a single animal.

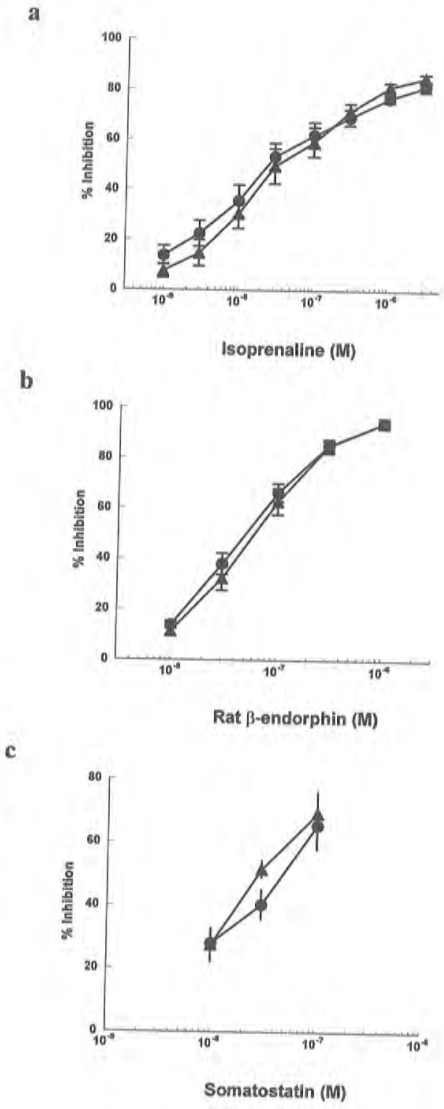

Figure 8.6: Concentration-response curves observed for isoprenaline (a), rat β-endorphin (b) and somatostatin (c) in the presence of PBS vehicle (circles) or MAb C4.19 at 60 μg/ml protein (triangles). The effects of drugs were expressed as % inhibition relative to stable pre-dosing contractions. Mean responses (n=6) are plotted with standard error bars.

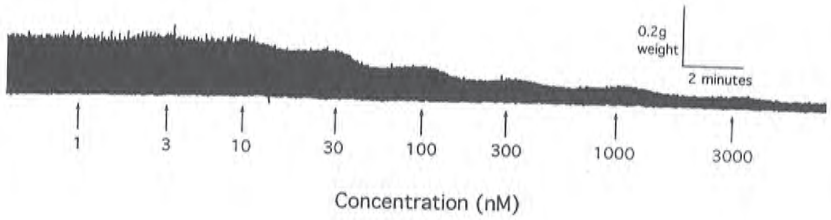

Figure 8.7: Original traces showing the inhibitory effect of isoprenaline on electrically-stimulated contractions of the isolated rat vas deferens. Cumulative concentrations of isoprenaline are shown.

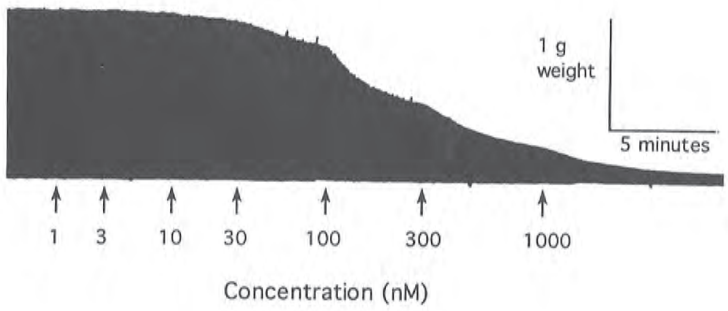

Figure 8.8: Original traces showing the inhibitory effect of rat β-endorphin on electrically-stimulated contractions of the isolated rat vas deferens. Cumulative concentrations of rat β-endorphin are shown.

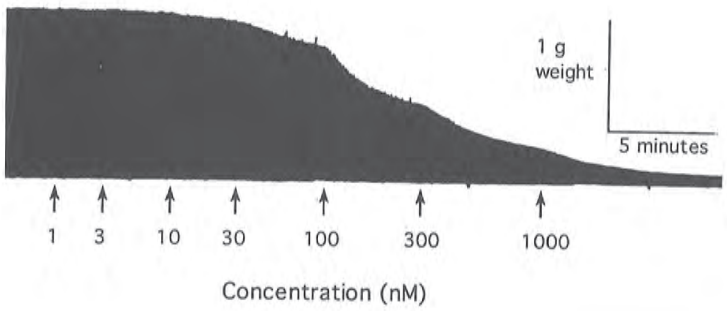

Figure 8.8: Original traces showing the inhibitory effect of rat β-endorphin on electrically-stimulated contractions of the isolated rat vas deferens. Cumulative concentrations of rat β-endorphin are shown.

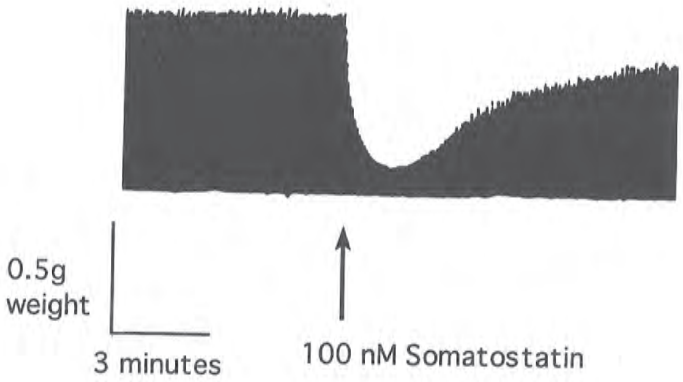

Figure 8.9: Original traces showing the inhibitory effect of somatostatin on electrically-stimulated contractions of the isolated rat vas deferens. Individual tissues were exposed to a single concentration because tachyphylaxis develops to the effect of somatostatin.

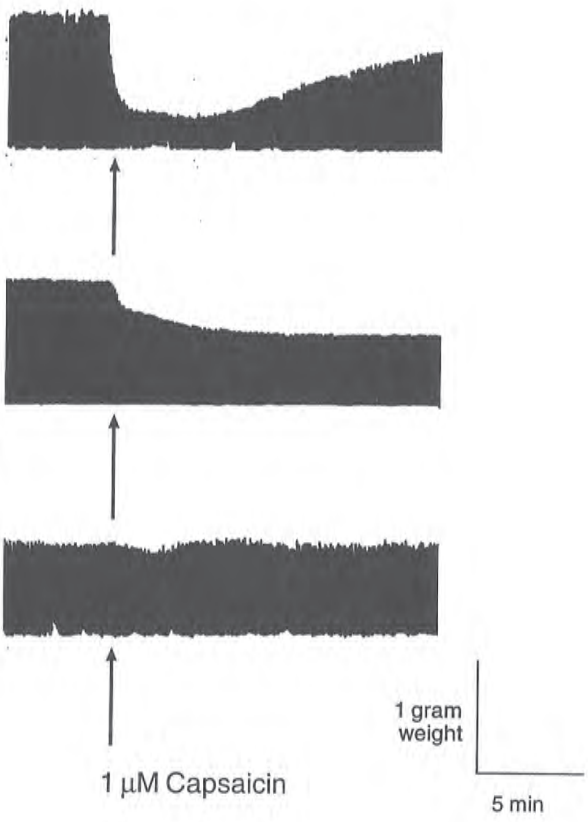

Figure 8.10: Original traces showing the inhibitory effect of 1 μM capsaicin on electrically-stimulated contractions of the isolated rat vas deferens in the presence of irrelevant control MAb directed against thyroid-stimulating hormone (top) or MAb C4.19 at 60 μg/ml protein (middle and bottom). The degree of immunoblockade was variable, with partial (middle) or almost complete (bottom) blockade of the capsaicin effect observed in different tissues compared with control.

Table 8.3: Mean effect of 1 μM capsaicin (95% confidence interval) in the presence of MAb C4.19 or control (% inhibition; n=6 to 8)

% Inhibition in presence of MAb C4.19 at 60 μg/ml	Control % inhibition (PBS vehicle[a] or irrelevant anti-TSH MAb at 60 μg/ml[b])	Mean MAb-Control difference (95% confidence interval)
26.0 (15.2 to 36.8)	60.8 (51.8 to 69.9)[a]	-34.8 (-22.6 to -47.0)*
36.1 (16.6 to 55.5)	67.0 (50.0 to 83.9)[b]	-30.9 (-14.3 to -47.5)**

* P=0.0003; ** P=0.005

8.3.3. Immunoblockade of endogenous CGRP

Capsaicin inhibited the electrically-stimulated contractions of the isolated vas deferens (Figure 8.10, Table 8.3). The response to 1 μM capsaicin was completely desensitized on second administration to tissues. This suggested that the response to capsaicin was due to a specific action on sensory nerves (Maggi *et al.*, 1987; Maggi & Meli, 1988). The effect of capsaicin was significantly attenuated by a 45-minute pre-incubation with MAb C4.19. Time-course experiments (Table 8.1) showed that an antibody incubation period of 45 minutes produced optimal immunoblockade. The peak effect of capsaicin was observed rapidly in the presence of vehicle, followed by a gradual recovery. The time to peak effect was slower and recovery more gradual in the presence of MAb C4.19. Blockade was evident when the response in every pair of tissue obtained from the same animal was compared. The blockade of the effect of capsaicin by MAb C4.19 was also significant compared with an irrelevant mouse anti-TSH MAb (Table 8.3). The mean responses to capsaicin in the presence of MAb C4.19 or control were in reasonable agreement with the magnitude of immunoblockade of exogenous CGRP responses.

8.4. Discussion

Several MAbs which block a biological effect of CGRP have been identified. The effects of MAb C4.19 have been characterized in detail, with particular reference to specificity of immunoblockade. Good agreement of observed and predicted

immunoblockade of exogenous CGRP with MAb C4.19 provided the basis for attempts to block endogenous CGRP released by capsaicin. The present findings suggest that endogenous CGRP released from primary afferent neurons is partly responsible for inhibition of nerve-mediated contractions of the rat vas deferens.

Immunoblockade has been used to investigate the role of endogenous CGRP in mediating the effect of capsaicin in the rat vas deferens (Maggi *et al.*, 1987). However, a rabbit anti-CGRP serum was used, the blockade of exogenous RβCGRP was not characterized, and no attempt was made to model the dynamics of immunoblockade. The low dilution of antiserum (1 in 25) required for immunoblockade in this study raised issues of specificity. The authors proposed immunoblockade as a pharmacological tool to study the possible physiological roles of CGRP as a neurotransmitter. A further evaluation of immunoblockade as an experimental tool has been performed in the present study.

The binding of an antibody to a peptide does not always result in blockade of biological activity. As discussed in Chapter 2, some workers have reported potentiation of the biological effects of peptides by anti-peptide antibodies (Tilders *et al.*, 1990; Shaw *et al.*, 1992). This was not observed in the present investigation. Pharmacological screening of a panel anti-CGRP MAbs allowed selection of those that blocked both species of rat CGRP effectively. A MAb such as C4.19 which equipotently blocks the biological activity of both forms is likely to be more generally useful than MAbs which are selective for either form (e.g., MAb R2.73). It is unclear at present whether αCGRP or βCGRP is the major form found in the vas deferens. αCGRP appears to be the major form found in peripheral sensory nerves whereas βCGRP appears to predominate in enteric nerves (Mulderry *et al.*, 1988; Sternini & Anderson, 1992). However, both forms of CGRP are expressed in peripheral sensory nerves innervating many tissues (Noguchi *et al.*, 1990; Sternini & Anderson, 1992).

The time-course experiments showed that due consideration must be paid to antibody incubation periods. However, a distinction should be drawn between requirements for the blockade of exogenous CGRP added to the tissue bath and endogenous CGRP released into the synaptic cleft compartment. Antibodies which are effective in immunoblockade should achieve maximum binding of antigen within a time frame of seconds (van Oers & Tilders, 1991). Therefore it was not surprising that incubation of MAb C4.19 for longer than 10 minutes produced no enhancement of blockade of exogenous CGRP. No difference is expected because 10 minutes should be sufficient for the antibody to distribute homogeneously within the tissue bath and antibody concentration did not change with time. In contrast, an incubation time of 10 minutes was clearly inadequate to achieve blockade of the capsaicin response, indicating that more time must be allowed for MAb C4.19 to diffuse into

the synaptic cleft. The results of the time-course experiment suggested that the concentration of the antibody had reached equilibrium in the synaptic cleft after 45 minutes since incubation with MAb C4.19 for 90 minutes did not enhance blockade of the capsaicin response.

The use of MAbs does not obviate the need to demonstrate specificity. The very specificity of an MAb could result in cross-reaction with other peptides or proteins bearing the same epitope. The potential non-specific binding of the MAb C4.19 to CGRP was characterized by controlled experiments with a MAb directed against an irrelevant epitope (MAb MAR 18.5). The potential of the anti-CGRP MAb C4.19 to cross-react with other drugs and peptides producing similar pharmacological responses was assessed by experiments with isoprenaline, rat β-endorphin and somatostatin. Similarly, a non-specific blockade of endogenous CGRP released by capsaicin was excluded by an experiment using an irrelevant anti-peptide (TSH) MAb as a control. The specificity of MAb C4.19 was also demonstrated in immunocytochemistry experiments. Pre-incubation of MAb C4.19 with 2 μM RαCGRP for 90 minutes at room temperature completely abolished immunostaining in rat spinal cord sections (Chapter 4).

Apparent parallel rightward shifts in the CGRP concentration-response curves were observed in the presence of MAb C4.19. The magnitude of the rightward shift was predicted well by the binding and pharmacodynamic models. However, the theoretical curve was steeper because of the non-linear relationship between total and bound concentration of CGRP predicted by the antibody binding model. One reason for the discrepancy between observed data and theoretical prediction might be experimental error. Another explanation is the assumption of equilibrium conditions for antibody binding prior to consideration of receptor binding. However, the antibody has to compete with receptor for binding, initially under non-equilibrium conditions, when CGRP is added to the tissue bath. A steady-state, corresponding to stable pharmacodynamic response observed, is eventually achieved. Under non-saturating conditions, the proportion of CGRP bound to antibody or receptor is likely to depend on the relative on- and off-rates of antibody and receptor binding and the relative concentrations of antibody and receptor. Modelling of the competitive binding of CGRP to antibody or receptor clearly requires more complex mathematical treatment. However, the present analysis has shown that the assumption of an equilibrium antibody binding model is sufficient to explain the magnitude of the rightward shift in the CGRP concentration-response relationship in the presence of MAb C4.19. It is interesting to note that a steeper non-parallel rightward shift of the CGRP concentration-response relationship, similar to the one simulated here, has been observed in the presence of an anti-CGRP antiserum in the isolated guinea-pig

bladder base (Maggi *et al.*, 1988). However, a near-parallel shift of the CGRP concentration-response relationship was observed with the antiserum in the isolated rat vas deferens preparation (Maggi *et al.*, 1987).

Immunoblockade should be considered as a complementary tool to immunocytochemical, electrophysiological and pharmacological studies using receptor antagonists. CGRP-like immunoreactivity outflow from the rat vas deferens is increased 4.5-fold by 10 μM capsaicin (Santicioli *et al.*, 1988). This effect was desensitized on second application of capsaicin. Desensitization is a feature of the specific action of capsaicin on sensory nerves (Maggi & Meli, 1988). CGRP-immunoreactive nerves are found around the deferential artery, in the smooth muscle layer and in the lamina propria (Goto *et al.*, 1987). Pre-incubation with capsaicin *in vitro* causes disappearance of CGRP immunostaining in the rat vas deferens (Saito *et al.*, 1987). The capsaicin antagonist, capsazepine, blocks the effect of capsaicin on the electrically-stimulated contractions of isolated rat vas deferens but not the inhibitory effect of CGRP (Maggi *et al.*, 1993).

Several lines of evidence point to a direct effect of CGRP on smooth muscle rather than a prejunctional effect on sympathetic nerves. RαCGRP does not alter the uptake or release of tritiated noradrenaline in mouse vas deferens (Al-Kazwini *et al.*, 1987). In electrophysiological studies of transmural nerve stimulation in the isolated rat vas deferens, HαCGRP did not affect the amplitude of excitatory junction potentials which are associated with nerve excitation or transmitter release. In contrast, HαCGRP inhibited the contraction of rat vas deferens induced by direct stimulation of smooth muscles (Goto *et al.*, 1987). $H\alpha CGRP_{8-37}$ blocked the effect produced by a low concentration of capsaicin (30 nM) in the isolated rat vas deferens (Maggi *et al.*, 1991). The results of the present immunoblockade experiments, together with the complementary evidence reviewed, provide strong evidence that CGRP partly mediates the efferent function of capsaicin-sensitive primary afferent neurons in the rat vas deferens.

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CHAPTER 9

In vivo immunoblockade studies with an anti-CGRP monoclonal antibody and its Fab' fragment: role of CGRP as an endogenous vasodilator

9.1. Introduction

A major objective of the project was to demonstrate the utility of immunoblockade as a pharmacological technique for probing the role of CGRP as vasodilator. The investigations described in Chapter 8 have resolved important methodological issues concerning *in vitro* immunoblockade. MAb C4.19 was shown to be a highly effective antibody for immunoblockade experiments and the neurotransmitter role of CGRP was demonstrated in the isolated rat vas deferens. However, it remains to be established that immunoblockade is an effective experimental technique *in vivo*.

The pharmacokinetics of any drug must be considered in order to achieve optimal *in vivo* effects. Fab' fragments offer potential pharmacokinetic advantages over IgG (Chapter 2) but comparative studies of *in vivo* immunoblockade of endogenous neuropeptides with MAb IgG or Fab' fragments are lacking. The pharmacokinetic advantages of anti-digoxin Fab fragments over IgG are clearly translated into pharmacodynamic benefits of more rapid and less variable reversal of potentially fatal arrhythymias following digoxin overdose (Lloyd & Smith, 1978). Thus *in vivo* immunoblockade has been attempted with MAb C4.19 IgG and its Fab' fragment (Chapter 4) in the present investigation.

Exogenous CGRP is one of the most potent vasodilators in man and in all animal species studied (reviewed in Chapter 1). Some indication of the importance of CGRP in the regulation of blood flow has already emerged from studies with HαCGRP$_{8-37}$ which acts as a CGRP receptor antagonist (Chapter 1). The hypotensive response to exogenous CGRP in anaesthetized and conscious rats may be blocked by HαCGRP$_{8-37}$ (Donoso *et al.*, 1990; Gardiner *et al.*, 1990). Exogenous CGRP produces a sustained hypotension that mimics the depressor response to spinal cord stimulation in the pithed rat (Taguchi *et al.*, 1992). The hypotensive responses to spinal cord stimulation and exogenous CGRP are markedly inhibited by HαCGRP$_{8-37}$. Thus endogenous CGRP appears to be a major neurotransmitter that mediates neurogenic vasodilatation following spinal cord stimulation in the rat. HαCGRP$_{8-37}$ given by the i.v. route has been found to inhibit the increased skin blood flow induced by intradermal CGRP and capsaicin (Escott & Brain, 1993). Increased skin blood flow in the rat hind paw following antidromic stimulation of the saphenous nerve is also inhibited by HαCGRP$_{8-37}$ (Delay-Goyet *et al.*, 1992; Escott

& Brain, 1993). The evidence obtained from the use of HαCGRP$_{8-37}$ suggests that CGRP is an important mediator of the "efferent" vasodilatory function of capsaicin-sensitive primary afferent fibres.

The principal objective of the studies described in this chapter was to investigate the utility of *in vivo* immunoblockade as an alternative pharmacological strategy for probing the role of CGRP in the regulation of blood flow and blood pressure. The results of immunoblockade were compared to those obtained by receptor blockade with HαCGRP$_{8-37}$. The effectiveness of MAb C4.19 in blocking the blood pressure response to exogenous CGRP was first established. This was followed by attempts to block the increased skin blood flow response in the rat hind paw produced by antidromic stimulation of the saphenous nerve. A potential interaction between CGRP and substance P in the skin blood flow response to antidromic nerve stimulation was also investigated with receptor antagonists. Successful immunoblockade of antidromic vasodilatation led to an investigation of the effect of MAb C4.19 Fab' fragment on the pressor response to angiotensin II.

9.2. Methods

Equipment

Blood pressure monitor (Green Acre Electronics)

Blood pressure transducer model 840 (SensorNor, Horten, Norway)

Blood pressure measuring unit with pressure valve (Speidel and Keller, Germany)

Graphtec linearcorder WR3310 with bridge coupler and heart rate coupler

Chart recorder (Kipp and Zonen, Germany)

Grass S88 stimulator (Grass Instruments, Mass., USA)

SM2-2B microscope for dissection (Nikon)

MBF3D Laser blood flow meter (Moor Instruments)

Harvard homeothermic blanket control unit with rectal probe

Materials	**Supplier**
Guanethidine monosulphate	Sigma
RαCGRP (10^{-4} M stock solution)	Peninsula
HαCGRP$_{8-37}$ (10^{-4} M stock solution)	Bachem
Human angiotensin II (acetate salt; 1mg/ml stock solution)	Sigma
Substance P (acetate salt; 10^{-3} M stock solution)	Sigma
RP-67,580 (L733,036)	MSD
Pentobarbitone sodium (Sagatal; 60 mg/ml)	Rhone-Merieux
Pentobarbitone sodium (Expiral; 100 mg/ml)	Sanofi
Anti-CGRP MAb C4.19 IgG (21.1 mg/ml)	K. Tan

Anti-CGRP MAb C4.19 Fab' fragment (11.8 mg/ml)	K. Tan
Purified normal mouse IgG (technical grade)	Sigma
Normal mouse Fab' fragment (9.4 mg/ml)	K. Tan
Anti-thyroid stimulating hormone (TSH) MAb (MA/732.162)	Serono
Mouse anti-rat MAb MAR 18.5 (ATCC No. TIB 216)	ECACC, Porton Down

Stock solutions of peptides were prepared in 0.2% BSA solution to minimize non-specific binding to polypropylene tubes and stored at -20°C. All concentrations refer to free base concentrations except for guanethidine monosulphate which was freshly prepared at 5 mg/ml. RαCGRP and angiotensin II were diluted with normal saline to give the appropriate dose/kg body weight in 0.1 ml. Antibody preparations contained PBS as vehicle. The appropriate volume of stock solution was given with a Hamilton microlitre syringe. All other drugs were diluted in normal saline and the dose volume was 1 ml/kg. All drugs and antibody preparations were given by the i.v. route. The cannula was flushed with 0.1 ml normal saline following each injection.

9.2.1. Effect of exogenous RαCGRP on blood pressure

9.2.1.1. Experimental procedures

Male Sprague-Dawley rats (250-300 g) were anaesthetized by i.p. injection of pentobarbitone sodium (60 mg/kg). The trachea was cannulated (PP240 cannula; Portex) to maintain a clear airway. The left jugular vein and the right carotid artery were cannulated (PP50 cannula; Portex) for i.v. drug administration and blood pressure measurement respectively. In experiments using MAb C4.19 IgG, blood pressure and heart rate were measured via a blood pressure transducer (SensorNor Model 840, Horten, Norway) linked to a Graphtec linearcorder WR3310 with bridge and heart rate couplers. In all other experiments, blood pressure was measured by a blood pressure transducer linked to a Green Acre blood pressure monitor and recorded on a chart recorder (Kipp and Zonen, Germany). The blood pressure monitor was calibrated with a blood pressure unit with pressure valve (Speidel and Keller, Germany) prior to experiments. Body temperature was maintained at 37°C by the use of a rectal probe connected to a thermostatically-controlled heating blanket (Harvard homeothermic blanket control unit). The depth of anaesthesia was assessed by cardiovascular responses to pinching of a front paw. Anaesthesia was maintained by i.v. injection of 10 mg/kg pentobarbitone sodium when necessary.

9.2.1.2. Blockade of blood pressure response with MAb C4.19 IgG

Dose-response experiments with RαCGRP were performed following administration of 1 mg/rat MAb C4.19 IgG in the form of purified ascites. The initial choice of dose was based on effective blocking concentrations of this MAb of 20 μg/ml or higher in tissue bath experiments (see Chapter 8) and a rat extracellular fluid volume of about 300 ml/kg (Bianchi *et al.*, 1981; Johnston *et al.*, 1988) assuming that the MAb could distribute into such a space. Increasing doses of RαCGRP were given at 10 to 20 minute intervals. Blood pressure had always returned to baseline before the next dose was given. It was found that the response to 0.1 nmol/kg RαCGRP could be completely blocked 15 minutes following pre-treatment with 1 mg/rat MAb C4.19. However, it was considered that more time would be required for the distribution of the MAb to perivascular spaces for the blockade of endogenous CGRP. Since the objective was to demonstrate immunoblockade after a relevant period following MAb administration, dose-response experiments were performed 60 minutes following administration of 1 mg/rat MAb C4.19.

The potential effect of MAb C4.19 on the blood pressure response to substance P was investigated by giving 0.1 nmol/kg substance P before and 30 minutes after 1 mg/rat MAb C4.19.

9.2.1.3. Blockade of blood pressure response with MAb C4.19 Fab' fragment

The blockade of hypotensive responses to RαCGRP was assessed using 2 mg/rat MAb C4.19 Fab' fragment. The choice of dose was based on a comparison of the activity of the IgG and Fab' fragment in an indirect ELISA of RαCGRP (Figure 4.8b, Chapter 4) and the pharmacokinetic properties of Fab' fragments.

A test dose of 0.1 nmol/kg RαCGRP was given prior to injection of MAb C4.19 Fab' fragment. The dose-response relationship for the hypotensive effect of RαCGRP was then obtained, starting 15 minutes after administration of the Fab' fragment.

9.2.1.4. Blockade of blood pressure response with HαCGRP$_{8-37}$

Up to 3 test doses of 0.1 nmol/kg RαCGRP were given to each rat to check that no tachyphylaxis occurred with repeated doses. Given lack of tachyphylaxis, the effect of HαCGRP$_{8-37}$ was tested by comparing hypotensive responses to RαCGRP before and 3 minutes after 100 nmol/kg HαCGRP$_{8-37}$.

9.2.2. Change in skin blood flow measured by Laser Doppler flowmetry following antidromic stimulation of the saphenous nerve

9.2.2.1. Principles

Laser Doppler flowmetry is a non-invasive technique for the measurement of local tissue blood flow. The technique uses the Doppler shift of laser light. When light is reflected off a moving object its frequency is shifted, the amount of shift being dependent on the speed of the moving object. Laser light is used to illuminate the skin tissue resulting in some of the light being reflected straight back by the static tissue, and some being reflected by moving red blood cells. The reflected light from the static tissue and the frequency shifted light from moving red blood cells is collected and mixed in a photo-detector. The photocurrent can then be processed to produce an indication of the flux of the red blood cells.

The limitations of the laser Doppler technique must be appreciated in order to design experiments, minimize artefacts and analyse data appropriately. Many factors affect skin blood flow including ambient temperature, humidity and body position. External light sources, probe position, pressure and movement affect flux readings. The above factors were standardized as far as possible in the experiments described here.

The laser blood flow probe was calibrated before use with the motility standard provided by the manufacturer. Calibration was important for the linearizer to function correctly i.e. produce a flux signal that is linearly proportional to tissue blood flow.

9.2.2.2. Experimental procedures

Male Sprague-Dawley rats (240-300 g) were anaesthetized and cannulated for i.v. drug administration and blood pressure monitoring as for blood pressure experiments. Guanethidine (5 mg/kg) was given immediately following cannulation of the jugular vein to minimize vasoconstriction due to concomitant stimulation of sympathetic fibres of the saphenous nerve (Gamse & Saria, 1987). Thirty minutes later, the saphenous nerve of the right hind limb was carefully dissected from connective tissue with the aid of a microscope (model SM2-2B; Nikon) and cut proximally. The saphenous nerve was placed over platinum bipolar electrodes (positive electrode towards paw) and covered with white soft paraffin. The laser Doppler probe was secured with tape over the medio-dorsal side of the hind paw skin, which is the region innervated by the saphenous nerve. Skin blood flow, measured as blood cell flux, was monitored continuously with the Moor laser Doppler blood flow meter (MBF3D;

Moor Instruments) at a sampling rate of 1 Hz (flux time constant 1 second; concentration time constant 0.5 second). When a stable baseline flux (less than 5% variation) was established for at least 5 minutes, the distal end of the saphenous nerve was electrically stimulated with 20 pulses (2 Hz, 10 V, 1 millisecond, for 10 seconds by use of the Grass S88 stimulator). Saphenous nerve stimulation was repeated at 30 minute intervals or longer.

9.2.2.3. Quantification

The effect of saphenous nerve stimulation on hind paw skin blood flow was quantified in two ways:-

1) The maximum change in flux which may relate to the rate of release of CGRP and the maximum concentration of CGRP in the synaptic cleft.
2) The cumulative change in flux over time which may relate to the period over which CGRP is released following nerve stimulation and the duration of action of the CGRP released.

Details of data analysis are provided in the Section 9.2.4.

9.2.2.4. Reproducibility study

Prior to experimental studies, a reproducibility study was performed to assess the feasibility of between-group comparisons, the validity of using the first stimulation as a control within each animal and the sample size required to detect important differences.

The saphenous nerve was stimulated up to 5 times in each animal (n=4) at 30 minute intervals. The coefficient of variation (standard deviation divided by the mean followed by multiplication by 100) was calculated as a measure of variability.

9.2.2.5. Determination of sample size

In order to show a within-group mean difference significant at the 0.05 level (α; Type I error), with a power of 1-β where β is the Type II error, the number of rats required is:

$$2 \cdot \left[\frac{(z_\alpha + z_\beta) \cdot \sigma}{\delta} \right]^2$$

Derivation of the above formula is found in Armitage & Berry (1987). z_α is the z (standard normal deviate) value required for the chosen level of α. Assuming a

conventional choice of P=0.05, z_α is 1.96 for a two-sided test. z_β is the value of z required for the level of β. Since 1-β is the probability of detecting a difference as large as that specified under the alternative hypothesis, z_β is inherently one-sided. The one-sided z value corresponding to β of 0.20 (statistical power of 0.80) is 0.84. σ is the source population standard deviation which may be estimated from the sample standard deviation in the reproducibility study. δ is the pharmacologically important difference, under the alternative hypothesis, that one wishes to detect with a probability of 1-β.

It should be noted that, for small samples, the above calculation underestimates the sample size because it is based on the z-distribution. The *t*-distribution cannot be easily used for such calculations, since the value of *t* required for, say a *P* of 0.05, depends on the sample size (actually on *n*-1, the degrees of freedom), which is unknown (Kramer, 1988).

Based on the coefficients of variation estimated in the reproducibility study, it was calculated that 10 and 6 rats were required to detect percentage changes of 30% and 50% respectively significant at $P < 0.05$ with statistical power of 80%.

9.2.2.6. Effect of MAb C4.19 IgG

Immunoblockade was initially attempted 1 hour following 1mg/rat MAb C4.19 IgG which was highly effective at blocking the hypotensive response to exogenous RαCGRP. Preliminary experiments showed that 1 mg/rat of irrelevant MAb IgG (MAR 18.5) had no effect on the blood flow response 1 hour later compared with vehicle treatment. Further experiments were performed at a dose of 3 mg/rat MAb C4.19 IgG with antidromic nerve stimulation at 1 and 2 hours following IgG administration.

9.2.2.7. Effect of MAb C4.19 Fab' fragment

Vehicle or Fab' fragment (2 mg/rat) was given 30 minutes prior to nerve stimulation. Saphenous nerve stimulation was also performed 1 hour following injection Fab' fragment to test if a greater blockade could be obtained (n=2).

9.2.2.8. Effect of normal mouse Fab' fragment

Vehicle or normal mouse Fab' fragment (2 mg/rat) was given 30 minutes prior to nerve stimulation, as for experiments with MAb C4.19 Fab' fragment. A dose of 0.1 nmol/kg RαCGRP was given 40 minutes after normal mouse Fab' fragment to test whether blood pressure response to RαCGRP was affected.

9.2.2.9. Effect of HαCGRP$_{8-37}$

Vehicle or HαCGRP$_{8-37}$ (100 nmol/kg) was given 3 minutes before nerve stimulation. A 30 minute interval was allowed between control and test stimulations.

9.2.2.10 Effect of co-administration of HαCGRP$_{8-37}$ and RP-67,580

The neurokinin$_1$ (NK_1) antagonist RP-67,580 (10 mg/kg) and HαCGRP$_{8-37}$ (100 nmol/kg) were given 15 minutes and 3 minutes before nerve stimulation respectively. A 30 minute interval was allowed between control and test stimulations.

9.2.3. Effect of MAb C4.19 Fab' fragment on the pressor response to angiotensin II

The potential role of endogenous CGRP released in response to a pressor insult was investigated by testing the effect of MAb C4.19 Fab' fragment on the pressor response to angiotensin II. Two doses of 100 ng/kg angiotensin II were tested to check if tachyphylaxis occurred prior to administering sequential rising doses of 10, 30, 100 and 300 ng/kg. Responses to the two doses of 100 ng/kg were not significantly different ($p=0.41$; $n=6$). A second dose-response study was started 30 minutes following 2 mg/rat MAb C4.19 Fab' fragment.

9.2.4. Data analysis

Mean arterial pressure (MAP) was estimated by the following formula:

$$\frac{(2 \times \text{Diastolic BP}) + \text{Systolic BP}}{3}$$

where BP is blood pressure. The maximum MAP responses to drugs are reported.

Maximum change in skin blood flow attributable to nerve stimulation (Fmax) was expressed as the difference between maximum flux recorded after nerve stimulation and mean flux over 1 minute prior to stimulation. Cumulative change in skin blood flow was estimated by the area under the flux-time curve attributable to nerve stimulation (AUC) calculated as the difference between the area under the flux-time curve 5 minutes following nerve stimulation and the rectangular area due to baseline flux over the 5 minutes before nerve stimulation. Data was analysed by use of the software (version 79; Moor Instruments) in the laser Doppler flowmeter.

Absolute values were used to compare Fmax and AUC between stimulations obtained in each animal. A percentage change in the above parameters was calculated by comparing values obtained following drug administration with those obtained after vehicle control. The *t* test for paired data was performed for statistical comparison of responses to nerve stimulation within each animal. The unpaired *t* test was used to compare percentage changes between animals. The statistical analyses described above were performed by use of the Statgraphics-Plus package (STSC, Inc., Rockville, U.S.A.).

Data from the angiotensin II experiments were analysed by repeated measures analysis of variance which accounts for the correlation of responses from the same animal. This analysis was performed by use of the SAS System (Version 6; SAS Institute Inc., Cary, NC, U.S.A.).

A *P* value of less than 0.05 was considered statistically significant. Ninety-five percent confidence intervals (95% C.I.) were calculated to indicate the precision of estimates.

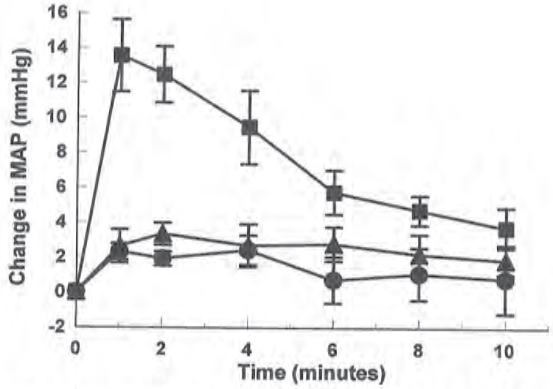

Figure 9.1: Effect of 1 mg/rat (circles) or 3 mg/rat (squares) i.v. MAb C4.19 IgG and HαCGRP$_{8-37}$ (100 nmol/kg i.v.; triangles) on baseline MAP. Mean results are plotted with standard error bars (n=4 to 6)

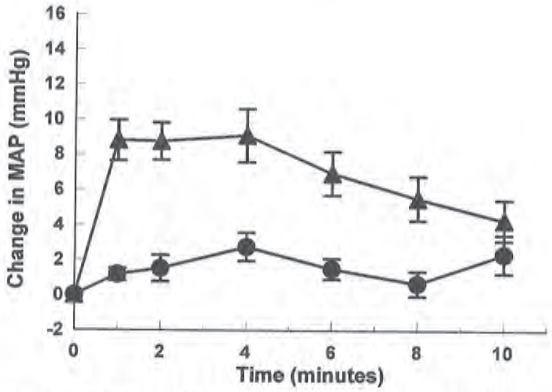

Figure 9.2: Effect of MAb C4.19 Fab' fragment (2 mg/rat i.v.; triangles) and normal mouse Fab' fragment (2 mg/rat i.v.; circles) on baseline MAP. Mean results are plotted with standard error bars (n=6)

9.3. Results

9.3.1 Effects on baseline MAP

HαCGRP$_{8-37}$ (100 nmol/kg) and 1 mg/rat MAb C4.19 IgG increased baseline MAP slightly but significantly (3.3 and 2.4 mmHg respectively; Figure 9.1). Increasing the dose of MAb C4.19 IgG to 3 mg/rat raised MAP by 13.5 mmHg (95% C.I. 7.7 to 19.3; P=0.02). A maximum response was observed at 1 minute followed by gradual recovery over 10 to 15 minutes (Figure 9.1). MAb C4.19 Fab' fragment (2 mg/rat)

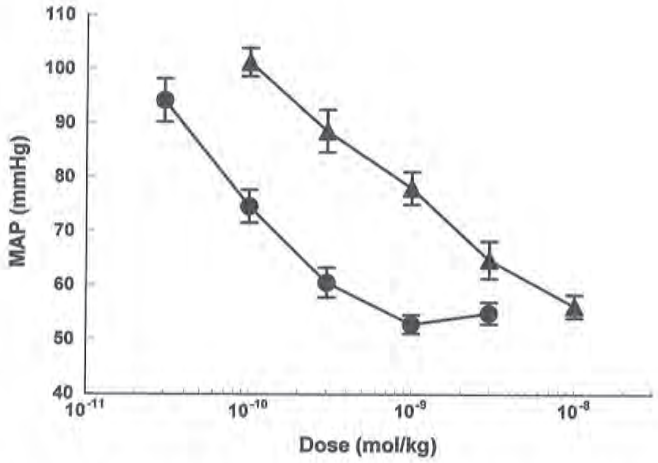

Figure 9.3: Effect of 1 mg/rat i.v. MAb C4.19 IgG (triangles) or 1 mg/rat i.v. control MAb IgG (circles) on the dose-response relationship for the effect of i.v. RαCGRP on MAP. Control MAbs were anti-TSH MAb or mouse anti-rat MAb MAR 18.5. The baseline MAP was not significantly different between the two groups. Mean results are plotted with standard error bars (n=6).

increased MAP by 9.1 mmHg (95% C.I. 5.4 to 12.8; P=0.02) but the same dose of normal mouse Fab' fragment did not increase MAP significantly. Like the whole IgG, the MAP increase due to MAb C4.19 Fab' fragment reached a maximum at 1 minute, with recovery within 10 to 15 minutes (Figure 9.2).

9.3.2 Blockade of the effect of RαCGRP on MAP

9.3.2.1. Blockade of MAP response with MAb C4.19 IgG

The dose-response relationship for the effect of RαCGRP on MAP was markedly shifted to the right 60 minutes following treatment with 1 mg/rat MAb C4.19 IgG compared with treatment with control MAbs (IgG) directed against irrelevant epitopes (Figures 9.3 and 9.4). The response to 0.1 nmol/kg RαCGRP was abolished following treatment with MAb C4.19 whilst the maximum response to RαCGRP at 1 nmol/kg (MAP 52.7 mmHg) was significantly blocked by MAb C4.19 (MAP 77.9 mmHg; P<0.001). The hypotensive response to RαCGRP was accompanied by a dose-dependent tachycardia (Figure 9.5). The heart rate response to 3 nmol/kg RαCGRP (487 beats per minute) was significantly blocked by MAb C4.19 IgG (442 beats per minute; P=0.03).

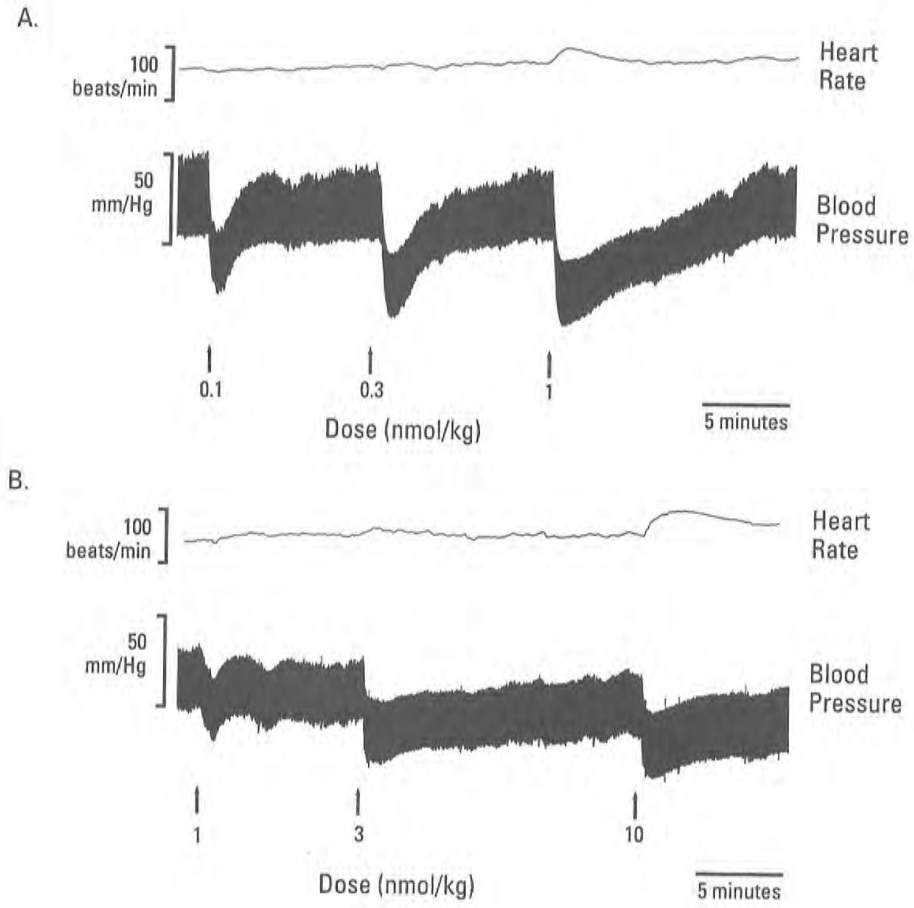

Figure 9.4: Original recordings of blood pressure and heart rate in a pentobarbitone-anaesthetized rat showing the effect of i.v. doses of RαCGRP following pre-treatment with 1 mg/rat irrelevant anti-TSH MAb IgG (a) or 1 mg/rat MAb C4.19 IgG (b). MAbs were given 60 minutes before the start of RαCGRP dosing.

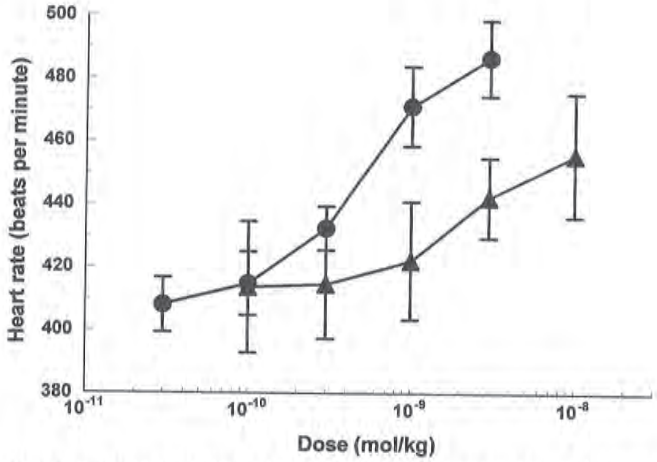

Figure 9.5: Effect of 1 mg/rat i.v. MAb C4.19 IgG (triangles) and 1 mg/rat i.v. control MAb IgG (circles) on the dose-response relationship for the effect of RαCGRP on heart rate. Control MAbs were anti-TSH MAb or mouse anti-rat MAb MAR 18.5. Mean results are plotted with standard error bars (n=6)

The MAP response to substance P 30 minutes following 1mg/rat MAb C4.19 IgG was not significantly different from the control response (Figure 9.6). The mean drop in MAP due to 0.1 nmol/kg substance P was 25.1 mmHg (95% C.I. 20.3 to 29.9) following MAb C4.19 IgG administration compared with the control mean response of 24.6 mmHg (95% C.I. 18.7 to 30.5).

9.3.2.2. Blockade of MAP response with MAb C4.19 Fab' fragment

The control dose of 0.1 nmol/kg RαCGRP decreased MAP by 29.5 mmHg (95% C.I. 21.7 to 37.3). This response was abolished in 5 out of 6 rats 15 minutes following 2 mg/rat of Fab' fragment. A partial blockade with a MAP drop of 10.5 mmHg was detected in one rat after dosing with Fab' fragment. However, the Fab' fragment, like the IgG, had no effect on the hypotensive response to 0.1 nmol/kg substance P (n=2). The dose-response relationship of RαCGRP was shifted to the right following administration of 2 mg/rat Fab' fragment to a similar extent as pre-treatment with 1 mg/rat IgG (Figure 9.7).

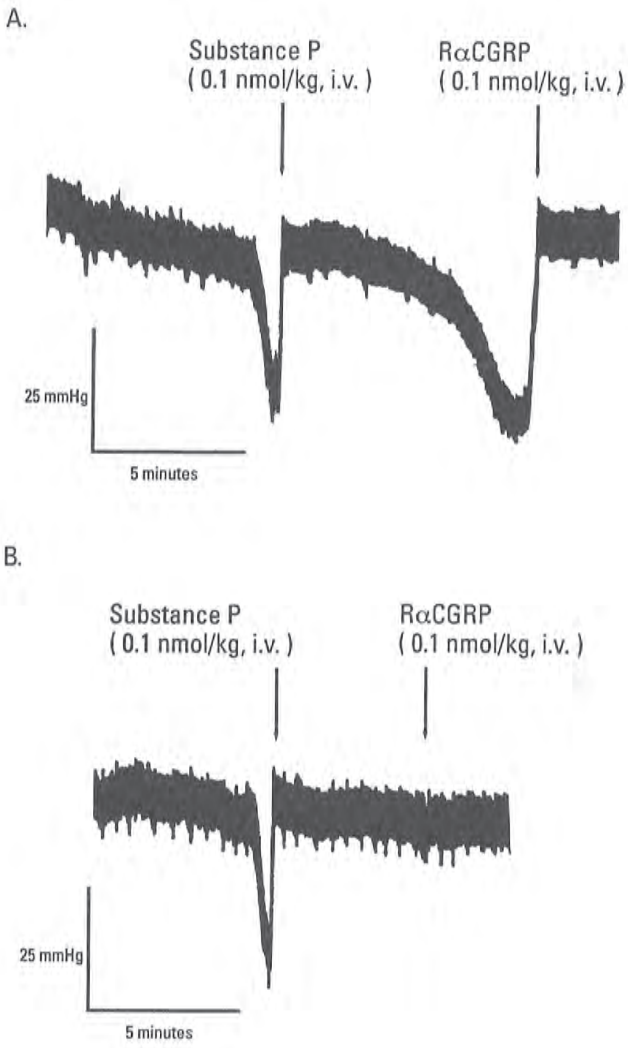

Figure 9.6: Original blood pressure recording showing control responses to 0.1 nmol/kg i.v. RαCGRP and 0.1 nmol/kg i.v. substance P (A). The response to RαCGRP was completely blocked but the response to substance P was unaltered 30 minutes following i.v. injection of 1 mg/rat MAb C4.19 IgG (B).

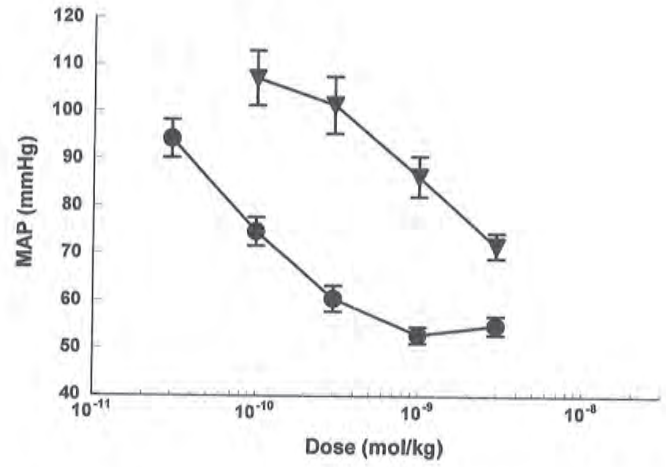

Figure 9.7: Effect of 2 mg/rat i.v. MAb C4.19 Fab' fragment (triangles) on the dose-response relationship for the effect of i.v. RαCGRP on MAP. The dose-response relationship of RαCGRP following treatment with 1mg/rat control MAb IgG (circles) is plotted for comparison. Mean results are plotted with standard error bars (n=6).

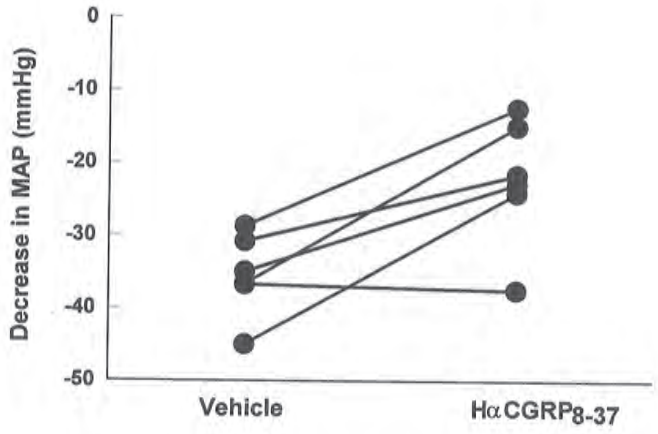

Figure 9.8: Effect of 100 nmol/kg i.v. HαCGRP$_{8-37}$ on the hypotensive response to 0.1 nmol/kg i.v. RαCGRP in 6 rats. HαCGRP$_{8-37}$ was given 3 minutes before RαCGRP.

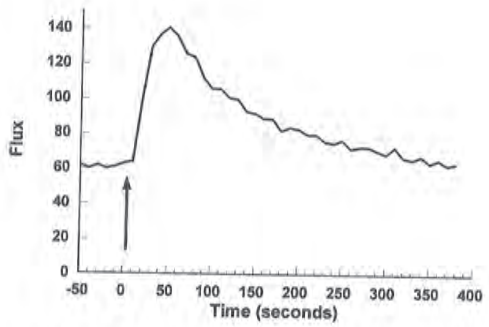

Figure 9.9: Typical change in skin blood flow following antidromic stimulation of the saphenous nerve (indicated by arrow) in a rat. Simulation parameters were 2 Hz, 10 V, 1 millisecond, for 10 seconds.

9.3.2.3. Blockade of MAP response with HαCGRP$_{8-37}$

The control dose of 0.1 nmol/kg RαCGRP decreased MAP by 35.5 mmHg (95% C.I. 29.5 to 41.5). This response was significantly attenuated by 100 nmol/kg HαCGRP$_{8-37}$ to 22.3 mmHg (95% C.I. 13.1 to 31.5; P=0.01; Figure 9.8).

9.3.3 Change in skin blood flow by measured by Laser Doppler flowmetry following antidromic stimulation of the saphenous nerve

9.3.3.1. Reproducibility study

Antidromic stimulation of the saphenous nerve produced a rapid increase in skin blood flow that remained elevated for 4 to 6 minutes (Figure 9.9). The results of the reproducibility study confirmed the early findings of Gamse & Saria (1987) with the laser Doppler technique. Figure 9.10a and 9.10b show that changes in Fmax and AUC were reproducible within each animal but responses varied widely between animals. However, between-animal comparisons could be made by calculating a percentage change from control stimulation in each animal.

The mean within-animal coefficient of variation for Fmax was 16.3%. The mean coefficient of variation for AUC was 20.2%. Importantly, there was no general trend towards a decrease or increase in Fmax or AUC with repeated stimulation.

9.3.3.2. Effect of MAb C4.19 IgG

A 1 mg/rat dose of MAb given 60 minutes prior to nerve stimulation did not block the skin blood flow response to antidromic nerve stimulation (n=2; Figures 9.11a and

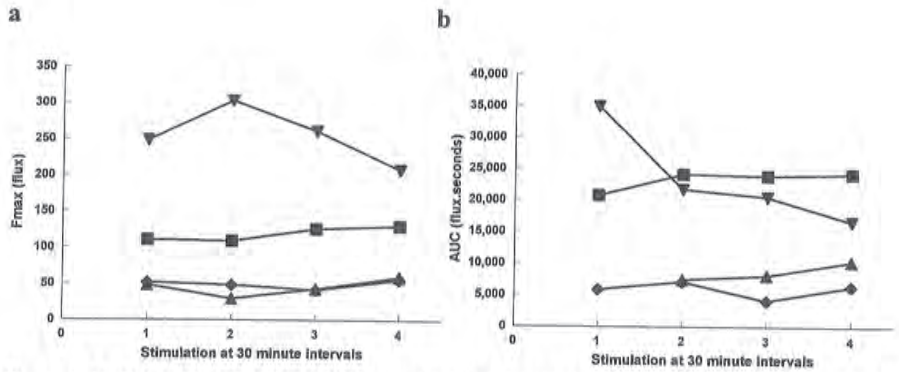

Figure 9.10: Fmax (a) and AUC (b) following repeated stimulation of the saphenous nerve in individual rats at 30 minutes intervals.

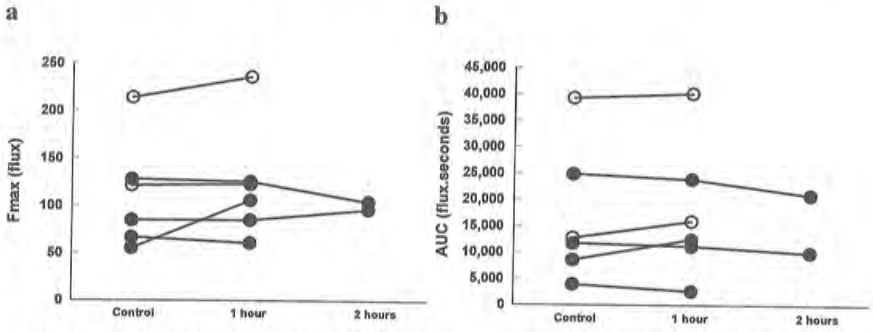

Figure 9.11: Effect of MAb C4.19 IgG at 1 mg/rat i.v. (open circles) or 3 mg/rat i.v. (closed circles) on Fmax (a) and AUC (b) in individual rats. Antidromic nerve stimulation was performed 1 or 2 hours following treatment.

9.11b). Increasing the dose to 3 mg/rat did not produce a significant difference in Fmax or AUC (P=0.83; n=4) after 60 minutes (Figures 9.11a and 9.11b). Further nerve stimulation performed at 2 hours following 3 mg/rat MAb produced slightly lower AUC compared with baseline stimulation, but not by more than 16% (n=2).

9.3.3.3. Effect of MAb C4.19 Fab' fragment

In contrast to experiments with whole IgG, the skin blood flow response to antidromic nerve stimulation was effectively blocked 30 minutes following administration of 2mg/rat MAb C4.19 Fab' fragment (Figures 9.12; Tables 9.1 and 9.2). AUC was blocked to a greater extent than Fmax. A greater blockade was not observed when nerve stimulation was performed at 1 hour following Fab' fragment administration (mean percentage decrease in AUC of 49.3% at 1 hour compared with 52.9% at 30 minutes; n=2).

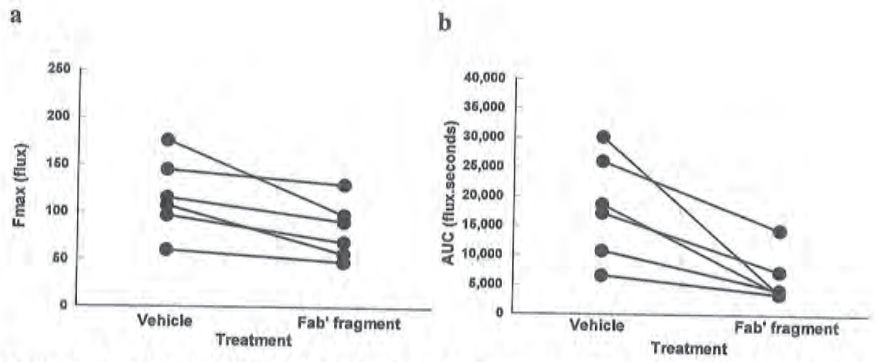

Figure 9.12: Effect of 2 mg/rat i.v. MAb C4.19 Fab' fragment on Fmax (a) and AUC (b) in individual rats. Antidromic nerve stimulation was performed 30 minutes following treatment.

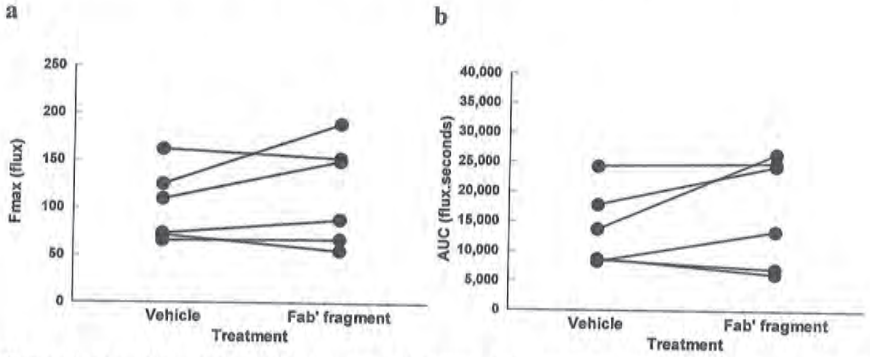

Figure 9.13: Effect of 2 mg/rat i.v. normal mouse Fab' fragment on Fmax (a) and AUC (b) in individual rats. Antidromic nerve stimulation was performed 30 minutes following treatment.

9.3.3.4. Effect of normal mouse Fab' fragment

Administration of 2 mg/rat normal mouse Fab' fragment 30 minutes prior to antidromic nerve stimulation increased the mean Fmax and AUC slightly but the changes were not statistically significant compared with vehicle control (Figures 9.13a and 9.13b; Tables 9.1 and 9.2).

Administration of 0.1 nmol/kg RαCGRP 40 minutes following 2mg/rat normal mouse Fab' fragment decreased MAP by 28.8 mmHg (95% C.I. 22.6 to 35.0), confirming a lack of immunoblockade (control hypotensive response 29.5 mmHg [95% C.I. 21.7 to 37.3]).

Table 9.1: Percentage change in Fmax attributable to the antidromic stimulation of the saphenous nerve following treatment with different drugs compared with vehicle control (n=6)

Drug	Mean % change in Fmax (95% confidence interval)	*P* value
MAb C4.19 Fab' fragment	-28.6 (-13.4 to -43.9)	0.02
Normal mouse Fab' fragment	13.8 (-15.3 to 42.8)	0.26
HαCGRP$_{8-37}$	-20.9 (-11.0 to -30.8)	0.003
HαCGRP$_{8-37}$ and RP-67,580	-57.1 (-44.4 to -69.8)	0.02

9.3.3.5. Comparison of the effects of MAb C4.19 Fab' fragment and normal mouse Fab' fragment

The mean percentage change in Fmax due to MAb C4.19 Fab' fragment was significantly different from that due to normal mouse Fab' fragment (Mean difference -42.4% [95% C.I. -70.8 to -14.0]; P=0.008). The mean percentage change in AUC due to MAb C4.19 Fab' fragment was also significantly different from that due to normal mouse Fab' fragment (Mean difference -87.6% [95% C.I. -133.7 to -41.5]; P=0.002).

9.3.3.6. Effect of HαCGRP$_{8-37}$

HαCGRP$_{8-37}$ at 100 nmol/kg significantly inhibited Fmax and AUC (Figures 9.14; Tables 9.1 and 9.2). Like MAb C4.19 Fab' fragment, HαCGRP$_{8-37}$ had a greater effect on AUC than Fmax.

Table 9.2: Percentage change in AUC attributable to the antidromic stimulation of the saphenous nerve following treatment with different drugs compared with vehicle control (n=6)

Drug	Mean % change in AUC (95% confidence interval)	*P* value
MAb C4.19 Fab' fragment	-62.9 (-44.5 to -81.3)	0.01
Normal mouse Fab' fragment	24.7 (-25.3 to 74.6)	0.20
HαCGRP$_{8-37}$	-74.6 (-56.2 to -92.9)	0.04
HαCGRP$_{8-37}$ and RP-67,580	-46.1 (-20.1 to -72.1)	0.08

9.3.3.7. Comparison of the effects of MAb C4.19 Fab' fragment and HαCGRP$_{8-37}$

The mean percentage changes in Fmax and AUC due to 2mg/rat MAb C4.19 Fab' fragment were not significantly different from those due to 100 nmol/kg HαCGRP$_{8-37}$ (P=0.30 and 0.27 respectively).

9.3.3.8. Co-administration of HαCGRP$_{8-37}$ and RP-67,580

The effect of co-administration of the two antagonists above on Fmax and AUC is plotted in Figures 9.15 and summarized in Tables 9.1 and 9.2. Co-administration of the two antagonists decreased Fmax to a significantly greater extent (P<0.001) compared with HαCGRP$_{8-37}$ alone. However, the percentage decrease in AUC with two antagonists was not greater than that observed with HαCGRP$_{8-37}$ alone. Indeed, the blockade was significantly poorer with two antagonists (P=0.01) although it should be noted that the 95% C.I. for the effect of one or two antagonists overlapped.

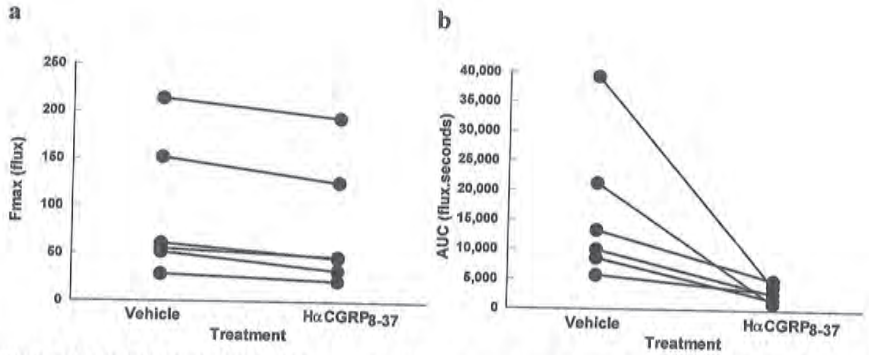

Figure 9.14: Effect of 100 nmol/kg i.v. HαCGRP$_{8-37}$ on Fmax (a) and AUC (b) in individual rats. HαCGRP$_{8-37}$ was given 3 minutes before antidromic nerve stimulation.

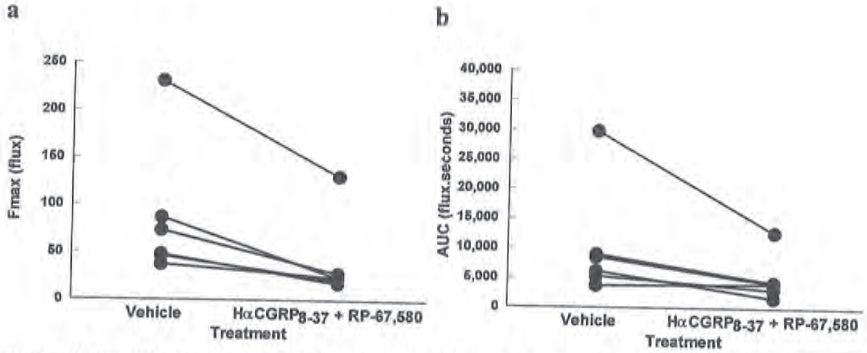

Figure 9.15: Effect of co-administration of 100 nmol/kg i.v. HαCGRP$_{8-37}$ and 10 mg/kg i.v. RP-67,580 on Fmax (a) and AUC (b) in individual rats. HαCGRP$_{8-37}$ and RP-67,580 were given 3 minutes and 15 minutes before antidromic nerve stimulation respectively.

9.3.4 Effect of MAb C4.19 Fab' fragment on the pressor response to angiotensin II

Administration of MAb C4.19 Fab' fragment did not significantly affect the dose-pressor response relationship (P=0.25) or the dose-recovery time relationship (P=0.40) of angiotensin II compared with vehicle control (Figures 9.16a and 9.16b respectively)

9.4. Discussion

This study has clearly demonstrated the ability of MAb C4.19 IgG and its Fab' fragment to block the hypotensive effects of exogenous RαCGRP *in vivo*. However, only the Fab' fragment was found to be an effective tool for the blockade of the

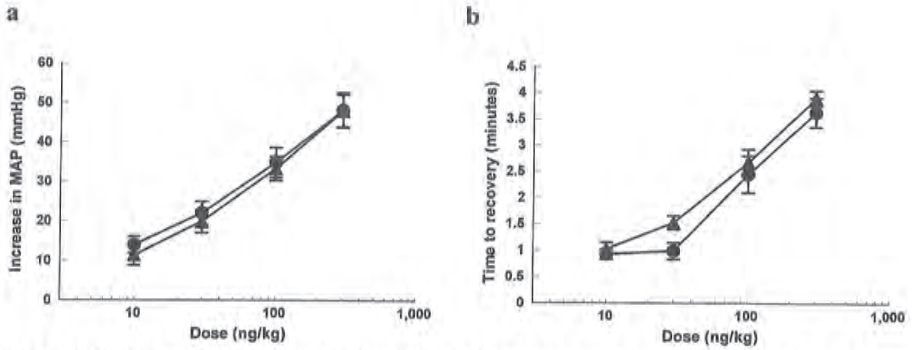

Figure 9.16: Effect of 2 mg/rat i.v. MAb C4.19 Fab' fragment (triangles) or vehicle (circles) on pressor response to i.v. angiotensin II (a) and time to recovery of MAP following pressor response to angiotensin II (b). Mean results are plotted with standard error bars (n=6).

increased hind paw skin blood flow following antidromic stimulation of the saphenous nerve. The results of the immunoblockade studies were in agreement with those obtained by the use of the receptor antagonist HαCGRP$_{8-37}$.

9.4.1. Immunoblockade of the MAP response to exogenous RαCGRP

MAb C4.19 IgG and Fab' fragment clearly shifted the dose-response relationship for the effect of RαCGRP on MAP to the right. Although the heart rate response was also blocked, it is not possible to distinguish between a direct chronotropic effect of RαCGRP or a reflex tachycardia from the present data. It has been demonstrated that RαCGRP increases cardiac output and reduces total peripheral resistance simultaneously in conscious rats (Lappe *et al.*, 1987). Therefore, the hypotensive effect of CGRP is mediated through peripheral vasodilatation rather than through reductions in cardiac output. The immunoblockade of the hypotensive response to exogenous RαCGRP served as the positive control for the immunoblockade of the vasodilatory effect of endogenous CGRP.

The dose of MAb may be expressed in binding sites. It was estimated by RIA (Chapter 4) that the amount of binding sites (B_{max}) of the MAb C4.19 IgG preparation was 1.3 nmol/mg. The observation that the 1 mg/rat dose of IgG preparation abolished the hypotensive response to 0.1 nmol/kg RαCGRP was not surprising since the amount of MAb binding sites given exceeded the exogenous dose of RαCGRP by about 50 times in a 250 g rat. The dose of RαCGRP that gave a maximal hypotensive response (1 nmol/kg; 250 pmol in a 250 g rat) was not sufficient to saturate the MAb binding sites, and blockade remained evident. However, a RαCGRP dose of 10 nmol/kg (2.5 nmol in a 250 g rat) exceeded the estimated

number of binding sites and produced an apparent maximal response in the presence of the MAb.

9.4.2. Increase in skin blood flow following antidromic stimulation of the saphenous nerve: immunoblockade of endogenous CGRP

Immunoblockade of the increased skin blood flow response produced by antidromic stimulation of the saphenous nerve strongly suggests that CGRP is a major neurotransmitter responsible for the neurogenic vasodilatation. The effects of MAb C4.19 IgG and Fab' fragment on antidromic vasodilatation were compared using doses that were approximately equipotent at blocking the hypotensive response to exogenous CGRP. The observation that only the Fab' fragment was effective at blocking endogenous CGRP pointed to a pharmacokinetic problem. The elimination of IgG would not be a limiting factor since the elimination half-life of heterologous IgG has been estimated to be longer than 2 days (Lloyd & Smith, 1978). Distribution of the antibody to the synaptic cleft is a prerequisite for the immunoblockade of endogenous neuropeptides. Given an adequate incubation period in a tissue bath, MAb C4.19 IgG clearly diffuses into the synaptic cleft since it was effective at blocking CGRP released from primary afferent nerves by capsaicin *in vitro* (Chapter 8). The most likely barrier to effective *in vivo* immunoblockade with IgG is a transport limitation due to poor capillary permeability (Chapter 2).

The observation that effective immunoblockade was achieved with MAb C4.19 Fab' fragment 30 minutes after administration whilst the IgG was ineffective up to 2 hours after the dose is consistent with reported antibody distribution characteristics. Covell *et al.* (1986) showed that the time to reach steady-state interstitial to plasma concentration ratio in the carcass (including muscle and skin) was 14 times more rapid for Fab' fragments than for whole IgG. Moreover, the steady-state interstitial to plasma concentration ratio in the carcass was 0.86 for Fab' fragments compared with 0.18 for whole IgG. Attempts were made in the present study to improve the likelihood of success with IgG by increasing the dose three-fold and doubling the time allowed for antibody distribution. The data of Covell *et al.* (1986) suggest that much larger doses and longer distribution time are required for successful immunoblockade with IgG. In this respect, it is interesting to note that Louis *et al.* (1989) reported significant immunoblockade of an increase in skin blood flow produced by topical application of mustard oil to the rat hind paw 60 minutes after injection of anti-CGRP PAbs. However, the response following CGRP antibody administration was not statistically compared with a rather similar response after non-immune rabbit serum. Nevertheless, the authors also observed immunoblockade of plasma extravasation with the anti-CGRP PAbs following topical mustard oil

application to the hind paw or application of capsaicin to the exposed saphenous nerve. A likely explanation for the discrepancy between the lack of effect of MAb C4.19 IgG and effectiveness of the anti-CGRP PAbs used by Louis *et al.* (1989) is leakage of IgG into interstitial space by plasma extravasation (Arfors *et al.*, 1979). The short stimulation period and mild stimulation parameters used in the present investigation would not have caused plasma extravasation (Escott & Brain, 1993).

The slow distribution of whole IgG to the site of immunoblockade could be overcome by the alternative strategies of active immunization with CGRP or chronic administration of IgG. Responses to stimuli that potentially release endogenous CGRP have been investigated in rats actively immunized with CGRP fragments conjugated to carrier proteins and compared with responses in rats immunized with carrier proteins alone (reviewed by Dockray *et al.*, 1992). Although successful immunoblockade by active immunization has been demonstrated for CGRP, such strategies are limited by antibody responses of variable affinity and specificity. Monitoring of antibody titres is required and it is necessary to start with relatively large numbers of animals.

With repeated administration, IgG should eventually distribute into interstitial space and achieve sufficiently high concentrations required for immunoblockade. A limited example is found in an investigation of the modulatory role of CGRP in chronic hypoxic pulmonary hypertension (Tjen-A-Looi *et al.*, 1992). Chronic infusion of CGRP for up to 16 days prevented pulmonary hypertension in hypobaric hypoxic rats whereas continuous infusion an anti-CGRP serum for the same period exacerbated pulmonary hypertension. However, the results could not be evaluated statistically due to the inadequate sample sizes (n=2).

9.4.3. Comparison of CGRP blockade by Fab' fragment or HαCGRP$_{8-37}$

Immunoblockade should be regarded as a technique that is complementary to the use of receptor antagonists. A comparison of the two approaches has been performed in this study. The blockade of antidromic vasodilatation by HαCGRP$_{8-37}$ was large and very similar to the blockade by the Fab' fragment; both HαCGRP$_{8-37}$ and the Fab' fragment blocked AUC more effectively than Fmax.. The magnitude of the inhibitory effect of 100 nmol/kg HαCGRP$_{8-37}$ on antidromic vasodilatation found in the present study (decrease in AUC of 74.6%) is in agreement with the findings of Delay-Goyet *et al.* (decrease in AUC of about 80%; 1992).

It has been noted in Chapter 1 that HαCGRP$_{8-37}$ may not be an ideal CGRP receptor antagonist. The utility of HαCGRP$_{8-37}$ as a receptor antagonist is sometimes limited by non-specific effects. For example, it has pro-inflammatory activity when

injected intradermally into rat skin (Brain *et al.*, 1992) and is capable of inducing histamine release from rat peritoneal mast cells *in vitro* (Hayes *et al.*, 1993). It has been suggested that the antagonism by HαCGRP$_{8-37}$ of HαCGRP-induced erythema in human skin is caused, not by receptor antagonism, but by an increase in the rate of degradation of HαCGRP by proteases released from skin mast cells by HαCGRP$_{8-37}$ (Hayes *et al.*, 1993). Following i.v. administration of HαCGRP$_{8-37}$, it is unclear whether a component of the blockade of antidromic skin vasodilatation could be due to increased CGRP degradation rather than CGRP receptor antagonism. If mast cell proteases are released by HαCGRP$_{8-37}$, the possibility of degradation of other neuropeptides that may be released by nerve stimulation cannot be excluded. Given these caveats, immunoblockade has provided useful supportive evidence that both the extent and pattern of blockade of antidromic skin vasodilatation by HαCGRP$_{8-37}$ reflect blockade of endogenous CGRP.

9.4.4. Co-administration of HαCGRP$_{8-37}$ and RP-67,580

The co-existence of substance P and CGRP in the peripheral nervous system has been clearly described (e.g., Lee *et al.*, 1985; see Chapter 1). Substance P and CGRP have been found in approximately 30% and 50% of the afferent neurons innervating the dorsomedial surface of the rat hind paw respectively (Louis *et al.*, 1989). Substance P has been implicated as a mediator of antidromic vasodilatation in the rat hind limb since the original report by Lembeck & Holzer (1979) that infusion of substance P into the femoral artery produced an increased outflow from the femoral vein. Investigations into the physiological and pathophysiological roles of substance P have been facilitated recently by the discovery of non-peptide neurokinin$_1$ (NK$_1$) antagonists. RP-67,580 is a potent NK$_1$ antagonist in the rat (Garret *et al.*, 1991; Beaujouan *et al.*, 1993). It has been found to block plasma extravasation (Garret *et al.*, 1991) but not the increased skin blood flow response (Delay-Goyet *et al.*, 1992) produced by antidromic stimulation of the saphenous nerve. Antidromic vasodilatation was not blocked significantly after a dose of up to 10 mg/kg RP-67,580 following nerve stimulation (D. Cook, personal communication; see Shepheard *et al.*, 1992). The objective of the present investigation was to examine a potential interaction between substance P and CGRP.

The greater blockade of Fmax with RP-67,580 and HαCGRP$_{8-37}$ than HαCGRP$_{8-37}$ alone suggests that substance P may have a transient but additive effect with CGRP as a vasodilator. This agrees with the transient hypotensive effect of substance P compared with CGRP. Although substance P and the NK$_1$ agonist GR73632 have little vasodilatory activity when injected intradermally into rat skin

(Richards *et al.*, 1993), RP-67,580 has been reported to block vasodilatation in the dorsal skin of the rat hind paw induced by close arterial injection of substance P-methyl ester into the femoral artery (Amman & Donnerer, 1993). The reasons for the conflicting evidence is unclear.

The finding that blockade of AUC was not augmented but, instead, diminished was somewhat surprising. However, substance P may have a role in regulating the vasodilatory activity of CGRP by stimulation or release of mast cell proteases which degrade CGRP (Brain & Williams, 1988). It could be hypothesized that antagonism of substance P has prevented the degradation of CGRP. The effect of HαCGRP$_{8-37}$ was therefore apparently diminished in the presence of the RP-67,580. The role of NK_1 receptors in substance P-induced mast cell degranulation is, however, unclear. It was thought that the basic N-terminus of substance P is involved in mast cell activation via a non-receptor process while the carboxyl terminus interacts with specific vascular receptors (Foreman *et al.*, 1983). However, further studies with substance P fragments suggest that both the N-terminal amino acids and the lipophilic C-terminal portion of the molecule are essential for histamine release from mast cells. The low activity of a range of neurokinin agonists compared with substance P suggest that the mast activation site is distinct from neurokinin receptors described in smooth muscle (Lowman *et al.*, 1988). Nevertheless, the observations in the present study should prompt an investigation into the possible effect of RP-67,580 on substance P-induced mast cell degranulation.

Substance P may not play a major role in mediating antidromic vasodilatation in rat skin but important regional or species differences should be considered. For example, Piedimonte *et al.* (1993) recently showed that substance P but not CGRP produced airway vasodilatation in the rat and that CP-99,994, an NK_1 antagonist, completely abolished the neurogenic vasodilatation induced by capsaicin in the rat airways. However, much more work must be performed to establish the role of substance P as a mediator of neurogenic vasodilatation, particularly in the light of new information that some non-peptide NK_1 antagonists, including RP-67,580, may exert non-specific inhibitory effects on neurotransmission (Wang *et al.*, 1994).

9.4.5. Effect of MAb C4.19 Fab' fragment on the pressor response to angiotensin II

Investigation of the role of endogenous CGRP requires some method of stimulating CGRP release from primary afferent fibres. Capsaicin has been used thus far for this purpose. Intravenous administration of capsaicin to Sprague-Dawley rats leads to a triphasic blood pressure response consisting of a fall, return to baseline or slight rise and a further fall in blood pressure (Donnerer & Lembeck, 1982). However, the

depressor response to capsaicin may not be primarily due to CGRP released from perivascular nerves. A reflex fall in blood pressure was observed following intra-arterial injection of capsaicin into the rat hind paw. Donnerer & Lembeck (1983) demonstrated that the afferent limb of this reflex consists of capsaicin-sensitive C-fibres, the reflex centre is located in the brain stem and the efferent pathway consists of a loss of sympathetic vasoconstrictor tone. Therefore immunoblockade was not used to investigate the role of endogenous CGRP released by capsaicin. Rather, consideration was given to physiological conditions under which an endogenous vasodilator may be released.

The angiotensin II converting enzyme inhibitor captopril has been shown to enhance the renal vasodilatory effects of CGRP in conscious rats (Bennett *et al.*, 1989). Studies with a angiotensin II receptor antagonist also suggest that CGRP-induced hypotension leads to activation of the renin-angiotensin system with secondary angiotensin II-mediated renal vasoconstriction (Gardiner *et al.*, 1991). It was hypothesized that the CGRP, in turn, may be released in response to counter-regulate pressor responses due to angiotensin II. To this end, I investigated whether MAb C4.19 Fab' fragment could potentiate the pressor response to angiotensin II through blockade of CGRP released as physiological regulatory mechanism. The Fab' fragment was used in preference to HαCGRP$_{8-37}$ in these experiments on the basis of longer half-life and more effective blockade of hypotensive responses to exogenous CGRP.

Angiotensin II is a potent direct vasoconstrictor. However, it also mediates the pressor response through interactions with other physiological systems, in particular, the sympathetic nervous system (Antonaccio & Wright, 1990). Noradrenaline released from sympathetic nerves by angiotensin II may in turn act to inhibit the release of CGRP through a presynaptic inhibitory effect on sensory neurons (Lundberg *et al.*, 1992; Kawasaki *et al.*, 1990). In addition, it is possible that angiotensin II could directly modulate CGRP release from perivascular nerves. Neurogenic release of CGRP-like immunoreactivity and vasodilatation induced by perivascular nerve stimulation of mesenteric artery is significantly decreased in spontaneously hypertensive rats. The reduced neurogenic vasodilatation mediated by CGRP-containing nerves is reversed by chronic treatment of spontaneously hypertensive rats with captopril (Kawasaki, 1992). Thus, the lack of effect of MAb C4.19 Fab' fragment on the pressor response to angiotensin II should be interpreted with consideration of potential interactions between the two vasoactive agents. The negative finding does not rule out a role for CGRP in the physiological regulation of blood pressure. Since the formulation of the original hypothesis and completion of these studies, Portaluppi *et al.* (1993) reported that i.v. infusion of angiotensin II led to dose-dependent

elevation of plasma CGRP levels in healthy volunteers. Thus the role of CGRP in the counter-regulation of pressor insults is worthy of further investigation, at least in man.

9.4.6. Effect of MAb C4.19 IgG and Fab' fragment on baseline MAP

The observation that MAb C4.19 IgG and Fab' fragment elevated baseline MAP suggested that circulating CGRP may have a role in the maintenance of vascular tone under resting conditions. The effect of the IgG was dependent on dose whilst no significant increase in MAP was observed after administration of normal mouse Fab' fragment. MAP was restored to baseline presumably because of compensatory mechanisms rather than disappearance of antibody since both IgG and Fab' fragment still blocked exogenous CGRP. A small but significant increase in MAP was observed after bolus injection of HαCGRP$_{8-37}$. These observations were principally made during antidromic vasodilatation experiments when blood pressure was monitored without simultaneous recording of heart rate. The effect of MAb C4.19 on basal blood flow *in vivo* must therefore be confirmed and the possibility of cross-reactivity with circulating vasoactive peptides with sequence homology to CGRP excluded. Although MAb C4.19 does not bind rat amylin (Chapter 4), cross-reactivity with adrenomedullin (Kitamura *et al.*, 1993) has not yet been tested.

Vasoconstrictor responses to CGRP blocking agents have been noted by others. Han *et al.* (1990a, 1990b) found that both a CGRP antiserum and HαCGRP$_{8-37}$ produced vasoconstriction in the isolated rat mesenteric vascular bed. HαCGRP$_{8-37}$ has been reported to constrict gastric submucosal arterioles (Chen *et al.*, 1992) and vasa nervorum (Zochodne & Ho, 1993). Gardiner *et al.* (1990) observed increased mean arterial pressure in conscious rats following infusion of HαCGRP$_{8-37}$ (300 nmol/kg/min) but this was accompanied by tachycardia. An increase in MAP following infusion of a lower dose of HαCGRP$_{8-37}$ (60 nmol/kg/min) was also observed by Taguchi *et al.* (1992) in the pithed rat.

Other data suggest that CGRP is not involved in the regulation of the resting tone. For example, Wei *et al.* (1992) did not observe any change in feline pial vessel diameter *in situ* on direct application of up to 1 μM HαCGRP$_{8-37}$. Lack of denervation hypersensitivity to CGRP in rats following destruction of primary afferent neurons by neonatal capsaicin treatment also argues against a sustained tonic influence of CGRP (Bachelard *et al.*, 1992; McEwan *et al.*, 1993). Nevertheless, variability in the role of CGRP in different vascular beds and different species cannot be excluded.

9.4.7. Application of MAb Fab' fragments in immunoblockade studies

In general, *in vivo* immunoblockade studies of CGRP have used PAbs. The present investigations have been performed with a MAb with inherent advantages of defined specificity, known affinity, reproducibility and unlimited availability. This study has demonstrated the superiority of Fab' fragments over IgG for the immunoblockade of endogenous CGRP following systemic administration. An advantage of goat polyclonal anti-CGRP Fab fragment over IgG in blocking capsaicin-induced inflammatory responses has also been demonstrated following intradermal injection into rabbit skin (Buckley *et al.*, 1992). The Fab fragment, but not IgG, inhibited the ability of capsaicin to potentiate oedema formation.

The fragmentation of whole IgG to Fab' fragments may be readily performed. Therefore the routine use of Fab' fragments should be advocated for acute *in vivo* immunoblockade experiments. Administration of Fab' fragments ensures the distribution of antibody to the site of immunoblockade. The interpretation of negative results is particularly difficult in immunoblockade studies because of the number of factors that must be considered. The use of Fab' fragments allows elimination of transport limitation as a potential explanation when findings are negative.

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CHAPTER 10

General discussion

The aim of the project was broad and encompassed the development and evaluation of new pharmacological tools and their application to the study of the role of CGRP as a vasodilatory neurotransmitter. The present effort has led to the achievement of 4 of the 5 specific objectives listed in Chapter 1 (Section 1.3). MAbs against CGRP were successfully developed and evaluated as tools for immunoblockade studies. The quantitative basis of immunoblockade as a pharmacological technique was established. The role of CGRP as a neurotransmitter was demonstrated *in vitro* in the neuroeffector junction of the rat vas deferens. *In vivo* immunoblockade with Fab' fragments of an anti-CGRP MAb showed that CGRP has a key role in mediating antidromic vasodilatation in the rat skin.

10.1. Critique of immunoblockade

10.1.1. *In vitro* evaluation of antibodies

Scientific answers to pharmacological questions depend on the availability, validity and reliability of pharmacological tools and techniques. Several basic criteria should be fulfilled for the valid use of immunoblockade as a pharmacological technique. Before antisera or MAbs are used for pharmacological experiments, they must be adequately characterized *in vitro*. Binding of antibody to antigen must be demonstrated in liquid phase. However, RIA results on their own are inadequate because the binding of an antibody to a ligand may not inhibit the binding of the ligand to its receptor. A radioligand binding assay is therefore more useful for the screening of immunoblocking antibodies. With an anti-peptide serum, inhibition of receptor binding may be mediated in part by internal image auto-anti-idiotypic antibodies which cross-react with the receptor.

A major purpose of RIA studies is to estimate the binding parameters of an antibody. The relevance of the antibody binding parameters Kd and B_{max} has been demonstrated theoretically (Chapter 2) and experimentally (Chapters 8 and 9) in this project. Unfortunately, they are rarely reported in immunoblockade studies. It is not sufficiently informative to report antibody binding parameters as dilutions giving a certain percentage binding of radioligand or amount of cold ligand (e.g., fmol/tube) required to inhibit binding by 50% (e.g., Maggi *et al.*, 1987). Likewise, it is difficult

to assess *in vivo* doses of purified PAbs[1] reported as being equivalent to volumes of original plasma (e.g., Louis *et al.*, 1989). In contrast, knowledge of the Kd and B_{max} of MAb C4.19 allowed prediction of the shift in concentration-response relationship in the isolated rat vas deferens assay and assessment of the extent of blockade of the hypotensive response to exogenous CGRP *in vivo*.

Recommendations are often made to modify RIA conditions to reflect conditions in which immunoblockade takes place, for example, performing RIA at 37°C in the presence of 10% serum in Krebs solution. However, it is quicker and more relevant to test antibodies directly in the pharmacological assay in which immunoblockade of an endogenous peptide is to be attempted. An *in vitro* pharmacological assay is the obvious starting point for the functional evaluation of immunoblockade.

10.1.2. Positive control experiments

Positive controls experiments using exogenous peptide must be performed in the relevant pharmacological test system. Peptide from the relevant species and multiple forms of a particular peptide, if present, should be tested. Consideration must be given to experimental design. Some experiments have been performed in which antibodies and peptides have been co-incubated prior to addition to a biological assay system (e.g., van Oers *et al.*, 1989). Such experimental conditions do not simulate the competition between antibody and receptor for the binding of a neuropeptide which has been rapidly released into the synaptic cleft. It is also inappropriate to claim immunoblockade of endogenous peptide *in vivo* when positive control experiments with exogenous peptide have been performed *in vitro*.

10.1.3. Specificity controls

Demonstration of the specificity of immunoblockade is critically important. When an antiserum is used, it is necessary to exclude the potential biological effects of serum components other than the antibodies directed against the peptide under study. An antiserum is often labelled as "specific" on the basis of lack of competitive binding of radiolabelled peptide by a range of other peptides. However, this does not control for the effect of various serum components on other potentially relevant signals. For example, lack of competition for radiolabelled CGRP binding to an antiserum by substance P does not exclude the presence of immunoglobulins in the serum which could bind substance P.

[1]"The term "polyclonal antibodies (PAbs)" is used instead of "antiserum" when purification has been performed.

In the case of MAbs, competitive binding studies do give some indication of specificity as this is primarily determined by the occurrence of homologous or similar epitopes on other molecules. In the immunoblockade of a neuropeptide, potential cross-reactivity of the MAb with other neuropeptides known to be co-released from stimulated nerves and which mediate the same biological response should be tested.

The most common negative control used for an antiserum is pre-immune or non-immune serum from the same species although occasionally specificity has been claimed on the basis of other proteins such as bovine γ-globulin (e.g., Uchida *et al.*, 1990). The injection of antigen in complete Freund's adjuvant could generate a heterogeneous response since *Mycobacterium tuberculosis*, the inflammatory agent of the adjuvant, is a polyclonal mitogen that may stimulate B lymphocytes unrelated to the response to antigen. The polyclonal response may include the production of anti-carbohydrate antibodies which react with membrane glycoproteins (Strosberg *et al.*, 1985). In addition, antibodies will be generated against carrier proteins used to conjugate low molecular weight antigens. Control sera have been reported to exhibit biological effects (Tilders *et al.*, 1990) and do not necessarily demonstrate specificity given that the control is often pre-selected for its inability to affect the biological parameter under study. Perhaps a better control would be serum from animals immunized according to an identical protocol but with carrier protein alone prepared in the same adjuvant. Such a control has been used in active immunization studies (e.g., Louis *et al.*, 1990)

By analogy to immunocytochemistry, an important specificity control for antisera is to remove the relevant antibody population and demonstrate disappearance of biological effect. This could be achieved by absorption of antibodies by incubation with biologically inactive peptide fragments or by affinity chromatography. Affinity chromatography with an antigen-coupled solid phase column has particular merits in that it simultaneously yields purified antigen-specific antibodies and a negative control in the form of the eluate. Moreover, antibodies may be concentrated during affinity purification.

The demonstration of dose-dependent effects of an antiserum or MAb also gives some hint of specificity because the lack of dose-dependency and the need for excessively high concentrations are warnings for non-specific effects.

Each of the controls considered above do not necessarily demonstrate specificity in isolation. Specificity is more convincing when several complementary controls are used.

10.1.4. Use of monoclonal antibodies and Fab' fragments

Immunoblockade studies require administration of large quantities of antisera to experimental animals. As a consequence, only a limited number of studies can be performed with a particular batch of antiserum. Batch to batch variation is of concern given that different antibodies may be inactive, enhance or block the effects of a particular peptide (for examples, see van Oers *et al.*, 1992). It is often difficult to reconcile qualitative and quantitative differences in results from studies utilizing antisera. The advantages of MAbs for immunoblockade have already been discussed in various contexts (e.g., Chapters 1, 8 and 9). A major reason for favouring MAbs for immunoblockade is a potentially unlimited supply of antibodies of defined specificity and affinity, thus ensuring reproducibility of results.

MAbs have limited potential disadvantages compared with PAbs. The specificity of a MAb could lead to major cross-reactions with other proteins bearing the same determinants whereas conventional antiserum would contain antibodies directed at determinants not common to the proteins and thus exhibit lower cross-reactivity. This may be relevant to the *in vivo* use of antibodies. Changes in the environment of an antigenic determinant, or in the way an antigen is presented, could alter the binding characteristics of an antibody - another potential disadvantage of the fine specificity of MAbs. It is also possible for PAbs to demonstrate higher functional affinity (avidity) due to the co-operative effects between multiple types of antibody. Subpopulations of antibodies against various determinants of a given antigen could contribute to avidity that would not be obtained with MAbs reacting with unique antigenic determinants. However, the disadvantages of MAbs may be circumvented by the use of appropriate selection procedures and experimental controls.

Whether MAbs or PAbs are used, Fab' fragments offer pharmacokinetic advantages. On the basis of the findings of this study and those of Buckley *et al.* (1992), Fab' fragments should be used in preference to whole IgG for *in vivo* immunoblockade. However, IgG is effective for *in vitro* immunoblockade provided that sufficient time is allowed for its diffusion to the site of action (synaptic cleft for a neuropeptide).

10.2. Immunoblockade of CGRP

Various workers have attempted to study the physiological role of CGRP by immunoblockade. Efforts which have led to positive results are summarized in Table 10.1. The first application of an anti-CGRP serum to block a biological effect of CGRP can be attributed to Lenz *et al.* (1984). Intravenous administration of the antiserum prevented the inhibitory effect of CGRP on gastric acid secretion following

Table 10.1: Summary of positive results on the immunoblockade of CGRP

Effect blocked	Reference
Inhibition of contractions of vas deferens	Maggi *et al.*, 1987
Nociceptive transmission	Kuraishi *et al.*, 1988
Urinary bladder motility	Maggi *et al.*, 1988
Expression of dopaminergic phenotypes	Denis-Donini *et al.*, 1989
Nociceptive transmission	Kawamura *et al.*, 1989
Neurogenic inflammation	Louis *et al.*, 1989
Suppression of growth hormone release	Fahim *et al.*, 1990
Vasodilatation (rat mesenteric arterial bed)	Han *et al.*, 1990
Contraction of guinea-pig trachea	Tschirhart *et al.*, 1990
Increase in cyclic AMP content of skeletal muscle	Uchida *et al.*, 1990
Gastric mucosal protection	Forster & Dockray, 1991
Skin vasodilatation/inflammation	Buckley *et al.*, 1992
Nociceptive transmission	Satoh *et al.*, 1992
Chronic pulmonary hypertension	Tjen-A-Looi *et al.*, 1992
Gastric mucosal protection	Lambrecht *et al.*, 1993
Postoperative gastric ileus	Zittel *et al.*, 1994

Note: active immunization studies which do not fall within the definition of immunoblockade (passive immunization) have been excluded.

i.v. but not intracerebroventricular administration. The study was an attempt to identify the site of action of centrally administered CGRP in mediating inhibition of gastric acid secretion rather than to demonstrate a physiological role for CGRP by immunoblockade.

Maggi and colleagues were the first to use immunoblockade to study the physiological role of CGRP *in vitro*. An antiserum against RαCGRP was shown to block CGRP- and capsaicin-induced inhibition of electrically-stimulated contractions of the isolated rat vas deferens (Maggi *et al.*, 1987) and the isolated guinea-pig bladder neck (Maggi *et al.*, 1988), thus implicating the involvement of CGRP as a neurotransmitter in these preparations. Effective immunoblockade was only achieved at a final dilution of 1 in 25 of the antiserum. The reported antibody binding parameters did not facilitate assessment of the immunoblockade observed. For example, since the concentration of binding sites (B_{max}) was not reported, it would appear that there was a major discrepancy between the reported antiserum dilution of 1 in 200,000 required for binding 40% of ^{125}I-RαCGRP by RIA and a dilution of 1 in 25 required for immunoblockade of RαCGRP with the same antiserum (Maggi *et al.*, 1987). In my experience, performing experiments in tissue baths containing Krebs

solution bubbled (very gently) with 95% O_2 and 5%CO_2 is difficult in the presence of serum at a final dilution of 1 in 500 or lower - considerable frothing occurs.

Satoh and colleagues used immunoblockade to investigate the role of endogenous CGRP in nociceptive transmission (Kuraishi *et al.*, 1988; Kawamura *et al.*, 1989; Satoh *et al.*, 1992). In contrast to the nociceptive threshold-lowering effect of intrathecally injected CGRP, antinociceptive effects were observed in rats following intrathecal administration of anti-CGRP sera. Minimal information was provided on the binding characteristics of the antisera used. The disadvantage of batch-to-batch variation of antisera was highlighted in these studies. The antinociceptive effect of one anti-CGRP serum in non-hyperalgesic rats (Kuraishi *et al.*, 1988) could not be reproduced with another anti-CGRP serum in a further study (Kawamura *et al.*, 1989).

Immunoblockade of an effect of CGRP within the brain has been attempted by Fahim *et al.* (1990). Injection of CGRP into the third ventricle of rats suppressed growth hormone and prolactin release. Intraventricular injection an anti-CGRP serum enhanced growth hormone release but had no significant effect on prolactin release. The antiserum used was labelled as "highly specific" on the basis of limited competitive binding data.

Denis-Donini (1989) used immunoblockade to investigate a longer term neurotrophic effect of CGRP. In the olfactory bulb, neurons only start to express tyrosine hydroxylase (leading to dopamine production) after olfactory epithelial cells have made synaptic contact. CGRP is produced by the olfactory epithelial cells and when the peptide is added to olfactory bulb neurons in culture, it induces the appearance of dopaminergic phenotype. When olfactory bulb neurons were co-cultured with epithelial neurons, the appearance of a dopaminergic phenotype was blocked by addition of an anti-CGRP serum. Control experiments were performed by washing off the antiserum; expression of dopaminergic properties was restored after a further period of cell culture.

RαCGRP and capsaicin contracts isolated guinea-pig tracheal strips. Tschirhart *et al.* (1990) reported that an anti-CGRP serum blocked the epithelial-dependent potentiation of capsaicin-induced contraction, and suggested that CGRP mediated the effect of capsaicin. The antiserum had unusual effects on the responses to exogenous RαCGRP; E_{max} was decreased but EC_{50} was not significantly altered. Indeed, in the presence of the antiserum, lower responses were observed with increasing concentrations of RαCGRP once a maximum effect had been achieved. Such observations are incompatible with simple competition between antibody and receptor for ligand binding. The specificity of the antiserum used must be questioned as the study did not otherwise include any controls, not even a non-immune serum.

The application of immunoblockade to the study of role of CGRP as a vasodilator has already been discussed in Chapter 9 and will only be briefly mentioned here. Han *et al.* (1990) showed that an anti-CGRP serum blocked the vasodilatory effects of exogenous CGRP and electrical nerve stimulation in the isolated perfused rat mesenteric arterial bed. Louis *et al.* (1989) reported that i.v. administration of anti-CGRP PAbs attenuated the increased skin blood flow response in the rat hind paw induced by topical application of mustard oil. The results of Louis *et al.* (1989) would be more convincing had the authors first demonstrated the specificity of the PAbs against exogenous CGRP *in vivo*. In rabbit skin, a polyclonal anti-CGRP Fab fragment was found to block increased blood flow responses induced by intradermal CGRP and capsaicin. Chronic infusion of CGRP into the pulmonary circulation of hypobaric hypoxic rats prevents the development of pulmonary hypertension. Tjen-A-Looi *et al.* (1992) showed that chronic infusion an anti-CGRP serum exacerbated pulmonary hypertension in this animal model. However, the effect of the antiserum on the responses to exogenous CGRP were not studied *in vivo* or in the isolated perfused lung studies reported.

In the isolated rat phrenic nerve-hemidiaphragm preparation, electrical stimulation of the phrenic nerve leads to CGRP immunoreactivity release and elevation of the cyclic AMP content in the diaphragm. Uchida *et al.* (1990) showed that an anti-CGRP serum could inhibit the increase in cyclic AMP content. No positive control experiments were performed with exogenous CGRP and bovine γ-globulin was used as the negative control.

Immunoblockade has been used to demonstrate the role of CGRP in gastric mucosal protection. Close arterial injection of anti-CGRP PAbs blocked the protective effect of exogenous CGRP and capsaicin on gastric mucosal damage induced by intragastric ethanol (Lambrecht *et al.*, 1993). Intravenous administration of anti-CGRP PAbs led to potentiation of the damaging effect of intragastric ethanol (Forster & Dockray, 1991).

Unlike the work on anti-CGRP MAbs described in this project, anti-CGRP PAbs have generally been used by others for immunoblockade. However, Wong *et al.* (1993) have reported the production of an anti-CGRP MAb that blocks somatostatin release, inhibition of gastric acid secretion and hypotension induced by exogenous CGRP. Antibody binding characteristics were reported in terms of titre of ascites fluid. The MAb has been successfully used to block the decrease in gastric corpus motility induced by abdominal surgery in rats (Zittel *et al.*, 1994). The authors suggested that endogenous CGRP mediated post-operative gastric ileus. However, the effect of the anti-CGRP MAb on CGRP-induced inhibition of gastric motility was not demonstrated.

The positive results of immunoblockade summarized in Table 10.1 led workers to conclude that CGRP is a physiologically important mediator of the various nerve-mediated responses. Conversely, negative results from attempts to block responses with anti-CGRP sera have been used to support the hypothesis of a lack of involvement of CGRP. Maggi *et al.* (1990) failed to block the relaxant effect of capsaicin on isolated strips of human colon with an anti-CGRP serum. However, the effect of the antiserum on the relaxant effect of exogenous CGRP was not demonstrated. Lefebvre *et al.* (1991) were unable to demonstrate immunoblockade of the relaxant effect of capsaicin on the isolated rat gastric fundus with an anti-CGRP serum. The antiserum (reported to be the same antiserum used by Maggi *et al.* (1987)) did not consistently block the effect of a single concentration of exogenous CGRP. Shiokawa *et al.* (1993) attempted to investigate the cerebrovascular role of CGRP in experimental subarachnoid haemorrhage in monkeys by intrathecal administration of anti-CGRP PAbs. Treatment with the PAbs led to a minor reduction in the baseline diameter of the major cerebral arteries but no significant effect on vessel diameter following subarachnoid haemorrhage. No positive control experiments were performed with the anti-CGRP PAbs. Five out of 9 animals died following intrathecal administration of the PAbs, making effective evaluation difficult. Thus the evidence for a lack of involvement of CGRP from the studies of Maggi *et al.* (1990), Lefebvre *et al.* (1991) and Shiokawa *et al.* (1993) is unconvincing due to the lack of, or inconsistent results from, positive control experiments.

The above review of the application of immunoblockade shows that the major shortcomings of many studies are the inadequate characterization of anti-CGRP sera used and the lack of appropriate controls. Without knowledge of antibody binding parameters, it is impossible to assess why the dilution of antiserum needed for immunoblockade should vary between 1 in 12.5 (Lefebvre *et al.*, 1991) and 1 in 16,000 (Han *et al.*, 1990). Lambrecht *et al.* (1993) identified two populations of antibodies with high and low affinity in their anti-CGRP serum and, unusually, reported Kd and B_{max} values. However, calculation of the ratio of B_{max} to Kd from their data revealed values of less than 1. Mathematical considerations in Chapter 2 show that B_{max} should exceed Kd by a factor of at least 10 for effective immunoblockade. Thus, the antibody binding parameters appear to be inconsistent with the positive immunoblockade results reported.

Some authors have attempted to demonstrate specificity of immunoblockade by showing lack of blockade following absorption of antisera with excess CGRP (Kurashi *et al.*, 1988; Kawamura *et al.*, 1989; Han *et al.*, 1990). Such control experiments may be confounded by the biological activity of remaining free CGRP; it is difficult to know whether lack of effect is due to neutralization of an antiserum or

simply functional antagonism of a non-specific effect of the antiserum by CGRP. A better approach would be to use inactive peptide fragments for absorption. For example, the enhancement of ethanol-induced haemorrhagic lesions by anti-CGRP PAbs was abolished following absorption of the PAbs with the C-terminal 28-37 fragment of CGRP (Forster & Dockray, 1991).

In vivo immunoblockade of endogenous CGRP in the gastrointestinal system has been achieved by the acute administration of IgG rather than Fab fragment (Forster & Dockray, 1991; Zittel *et al.,* 1994). The effective application of IgG for immunoblockade in the gastrointestinal tract compared with the skin (Chapter 9) could be explained by the differential distribution of the molecule to interstitial space in the gut. Covell *et al.* (1986) estimated that the steady-state ratio of interstitial to plasma concentration of IgG is 3.0 times higher and the time to reach steady-state 13.2 times quicker in the gut compared with the carcass.

The design of some of the immunoblockade studies with anti-CGRP antibodies reviewed may not be ideal but the studies compiled in Table 10.1 do illustrate the diverse applications of immunoblockade and its potential for probing the physiological role of CGRP.

10.3. Comparison of immunoblockade and receptor antagonism

A specific discussion of the relative advantages and disadvantages of immunoblockade and receptor blockade is dependent on the specific properties of anti-peptide antibodies and receptor antagonists in question. It may be argued that immunoblockade will provide little additional information compared with the ideal receptor antagonist. However, in practice, neuropeptide receptor antagonists are peptide analogues or fragments which may be metabolically unstable, lack specificity, possess intrinsic agonist activity, demonstrate variable selectivity for receptor subtypes or show low antagonistic potency (Regoli, 1985). The search for specific non-peptide antagonists often proves to be elusive. For example, excitement over the recent discovery of several non-peptide tachykinin receptor antagonists has been tempered by findings of non-specific effects on neurotransmission (Wang *et al.,* 1994).

In the absence of receptor antagonists, alternative pharmacological techniques must be sought for studying the physiological role of a neuropeptide. Alternative techniques include receptor desensitization, depletion of the peptide from nerves and immunoblockade. When a new peptide is discovered, immunoblockade may provide early evidence for its physiological role. It takes less time to raise antibodies against a

peptide than to produce conventional receptor antagonists or anti-receptor antibodies. Fragments of CGRP were identified and characterized as CGRP receptor antagonists about 6 years after the discovery of CGRP. HαCGRP$_{8-37}$ has proved to be an effective tool for studying the role of endogenous CGRP but, as reviewed in Chapter 1, it is far from the ideal receptor antagonist. Therefore, even when a receptor antagonist is available, immunoblockade may provide complementary information to receptor antagonism. This was clearly demonstrated in this project (Chapter 9) and in three other studies in which immunoblockade was used in conjunction with receptor blockade with HαCGRP$_{8-37}$ (Tjen-A-Looi *et al.*, 1992; Lambrecht *et al.*, 1993; Zittel *et al.*, 1994). Other results of immunoblockade studies have been independently confirmed with the use of HαCGRP$_{8-37}$, for example, the vasodilatory role of CGRP in the mesenteric arterial bed (Han *et al.*, 1990; Claing *et al.*, 1992).

Immunoblockade is not a substitute for the use of a receptor antagonist because it provides no information concerning the ligand-receptor interaction. A receptor antagonist that is specific for a receptor subtype will provide information on receptor heterogeneity that cannot be revealed by immunoblockade. This is well illustrated by the differential antagonistic potency of HαCGRP$_{8-37}$ in different tissues which has provided evidence for CGRP receptor heterogeneity. However, the very selectivity of a receptor antagonist may lead to failure to reveal an important physiological role in some tissues. For example, in the electrically-stimulated isolated guinea-pig bladder preparation, HαCGRP$_{8-37}$ at concentrations up to 3 μM did not block the inhibitory effect of CGRP (Giuliani *et al.*, 1992). In contrast, the inhibitory effect of CGRP and capsaicin on the electrically-stimulated contractions was blocked by an anti-CGRP serum (Maggi *et al.*, 1988). Evidence in support of a neurotransmitter role of CGRP was obtained despite the lack of a selective and potent CGRP receptor antagonist at the putative CGRP$_2$ receptor.

Immunoblockade is particularly useful when the results of receptor blockade studies are equivocal. HαCGRP$_{8-37}$ at 1 μM has been reported to both block and have no significant effect on CGRP-induced inhibition of electrically-stimulated contractions of the isolated rat vas deferens (Maggi *et al.*, 1991; Butler *et al.*, 1993). The immunoblockade studies performed in this project confirmed the neurotransmitter role of CGRP in the neuroeffector junction of the rat vas deferens.

Immunoblockade is usually defined in relation to inhibition of receptor binding. However, the technique may provide information on the role of CGRP independent of receptor binding inhibition. CGRP has been reported to potently inhibit the degradation of substance P by a substance P endopeptidase in cerebrospinal fluid (LeGreves *et al.*, 1985). Using the intrathecal route of administration, Mao *et al.* (1992) showed that CGRP enhanced substance P-induced behavioural changes in rats.

CGRP failed to enhance the effects of a substance P analogue that is resistant to degradation by substance P endopeptidase. The authors therefore suggested that the mechanism of this interaction is metabolic inhibition of substance P degradation by CGRP. If anti-CGRP antibodies inhibit the interaction of CGRP with substance P-degrading enzymes, the effect of substance P would be diminished. Blockade of the metabolic inhibitory role of CGRP could be an alternative explanation for the antinociceptive effects of anti-CGRP sera given intrathecally (Kuraishi *et al.*, 1988; Kawamura *et al.*, 1989; Satoh *et al.*, 1992).

10.4. Critique of anti-receptor monoclonal antibodies

10.4.1. Production of monoclonal antibodies against receptors

One of the objectives of the project was to develop MAbs which could act as competitive CGRP receptor antagonists. Screening procedures were oriented towards the detection of antibodies that could inhibit ligand binding in order to select those that may be directed to the receptor binding site. Practical experience suggests that antibodies directed against the binding site of some receptors are rare. For example, a review of several studies which reported the production of MAbs against the nicotinic receptor by immunization of mice with purified receptor showed that only 3 out of several hundred anti-receptor MAbs were directed to the cholinergic binding site (Strosberg & Schreiber, 1984).

A major difference in the production of anti-peptide MAbs and anti-receptor MAbs is that pure peptide, but not receptor, is readily available as antigen for immunization. Immunization with crude membrane preparations proved unsuccessful in this project. Considerable efforts were made by Wimalawansa *et al.* (1993) to purify the CGRP receptor from porcine cerebellum. Immunization of mice with the purified receptor led to the successful production of anti-receptor MAbs but the MAbs did not recognize the rat CGRP receptor (Chapter 6). The auto-anti-idiotypic strategy was therefore particularly attractive because it by-passes the need for receptor purification and may facilitate the generation of MAbs against the receptor binding site.

The value of the anti-idiotypic approach has been recently challenged by a study on the binding properties of a large number of MAbs directed against CD4, the receptor on T lymphocytes for the envelope glycoprotein gp120 of the human immunodeficiency virus (Davis *et al.*, 1992). From a panel of 225 anti-CD4 MAbs, only 10 MAbs bound to the same region as the natural ligand gp120. None of the MAbs had identical fine binding specificities and binding kinetics as gp120. In

addition, anti-idiotypic antibodies raised against a number of the anti-CD4 MAbs failed to bind gp120. Davis *et al.* (1992) therefore questioned the underlying concept of structural mimicry of receptors by antibodies. However, the lack of structural mimicry does not undermine the large body of evidence for the functional mimicry of ligands by anti-idiotypic antibodies (reviewed by Strosberg, 1989; Erlanger, 1991,1993(Erlanger, 1991; Erlanger, 1993)). Moreover, while exact structural mimicry may be an extremely rare phenomenon, there is evidence that peptide sequences from the complementarity-determining regions (CDRs) of anti-idiotypic anti-receptor antibodies are similar to natural peptide ligands (e.g., anti-idiotypic antibodies against the TSH receptor [Taub *et al.*, 1992]). It is noteworthy that the pharmacological objective of obtaining MAbs with receptor antagonistic properties does not require that such MAbs mimic the natural ligand. Indeed, receptor blockade may require quite different structural characteristics from the natural ligand. The anti-idiotypic approach has, nevertheless, led to the successful production of MAbs with receptor antagonistic activity (e.g., Costagliola *et al.*, 1991). Therefore, the findings of Davis *et al.* (1992), and the failure of the auto-anti-idiotypic approach in this project on methodological grounds, should not discourage further attempts to generate anti-CGRP receptor MAbs by this strategy.

The complementary peptide strategy (Chapter 1; Section 1.2.4.) could be pursued as an alternative method of generating anti-CGRP receptor antibodies, particularly as the nucleotide sequence of CGRP mRNA is known. However, the number of publications on the successful generation of anti-receptor antibodies by this strategy was small when the project was started. Successful production of antibodies against a variety of peptide receptors have now been reported (reviewed by Shabi *et al.*, 1992). This strategy may be worth pursuing on the basis of recent experimental evidence.

10.4.2. Pharmacodynamic properties of anti-receptor antibodies

When anti-receptor MAbs are identified, considerable efforts must be made to characterize their pharmacodynamic properties. In the identification of MAbs directed against the receptor binding site, MAbs should be tested for ability to displace binding of the ligand to its receptor. To exclude steric hindrance or non-competitive mechanisms, the converse (ligand displacement of anti-receptor MAb) must also be demonstrated. As for conventional ligands, saturability of MAb binding to receptor should be tested. The specificity of anti-receptor MAbs could be demonstrated in various ways but if the receptor gene has been cloned, receptor transfection studies may be performed to demonstrate unequivocally that binding is dependent on the

presence of the specific receptor (e.g., Taub *et al.*, 1992). Anti-receptor MAbs may act as agonists, partial agonists or antagonists. MAbs with antagonistic properties may be identified by the use of a functional assay such as activation of adenylate cyclase.

Anti-receptor MAbs may exhibit different properties to low molecular weight peptide or non-peptide antagonists. The bi- or multi-valency of antibodies may lead to aggregation or cross-linking of receptors, sometimes followed by internalization and processing of antibody-receptor complexes (Strosberg & Schreiber, 1984). *In vivo*, the binding of antibody to receptor may initiate Fc-mediated effector functions (complement activation, antibody-dependent cellular cytotoxicity) that may modify the biological effect triggered by the binding of antibody. Such effects may be avoided by the use of $F(ab')_2$ or Fab' fragments. Whilst there are many examples of biochemical applications of anti-receptor MAbs (e.g., biosynthesis, epitope mapping, receptor-response coupling, molecular cloning), their utility as *in vitro* and *in vivo* pharmacological tools remains to be clearly defined.

10.5. Future directions

The successful application of immunoblockade to demonstrate the role of CGRP as a vasodilatory neurotransmitter should encourage further application of the technique. It is likely that immunoblockade with anti-peptide MAbs will be applicable to the study of the physiological role of other neuropeptides. Positive immunoblockade results with anti-peptide sera have been reported for a range of neuropeptides including neurokinin A, substance P, vasoactive intestinal peptide, cholecystokinin and somatostatin (e.g., Maggi *et al.*, 1990; Grider *et al.*, 1985; Rozsa *et al.*, 1985). Like the immunoblockade studies with anti-CGRP antibodies reviewed above (Section 10.2.), some of these studies were inappropriately controlled. It is hoped that the criteria developed for immunoblockade studies in this chapter will be widely adopted.

Further development of the theoretical framework of immunoblockade is still required. There is a discrepancy between the theoretical prediction of lack of time for antibody binding during synaptic transmission (van Oers & Tilders, 1991) and the positive results reported for the immunoblockade of CGRP released by nerve stimulation in various neuroeffector junctions. Mathematical consideration of the competition between antibody and receptor for ligand binding is likely to require the application of compartmental modelling.

It is interesting to ask the questions of whether immunoblockade could be used to study the physiological role of CGRP in man and whether anti-CGRP antibodies have therapeutic potential. The possibility of answering both questions depend on the production of antibodies, fragments or mimetics that are not immunogenic in man.

The production of human MAbs from hybridoma technology has proved to be extremely difficult but recent advances in molecular biology are making widespread application of human antibodies an impending reality (Winter & Milstein, 1991).

Mouse MAbs such as MAb C4.19 may be humanized by transplanting the CDRs from mouse MAbs on to human antibody variable region frameworks (Verhoeyen *et al.,* 1988). In such "classical" antibody engineering, hybridomas of known specificity have provided the raw material for cloning the rearranged V_H and V_L genes. However, the development of rapid methods based on the polymerase chain reaction has allowed the cloning of V gene libraries from pools of B lymphocytes (Winter & Milstein, 1991). Complete antibody V domains can be displayed on the surface of fd bacteriophage; such phage antibodies can bind specifically to antigen (McCafferty *et al.,* 1990; Clackson *et al.,* 1991). Bacteria infected by phage antibodies are essentially converted to phage antibody "factories". Higher affinity phage antibodies may be obtained by random mutation of antibody genes (Hawkins *et al.,* 1992). The antibody genes can be rescued and used to produce soluble antibody fragments. Remarkably, Hoogenboom & Winter (1992) has constructed a human phage antibody library from the antibody genes of healthy blood donors, selected phage by binding to antigen, and obtained human antibody fragments with specific binding activities. Thus phage antibody technology allows immunization to be by-passed and offers a powerful route to the production of human MAbs. A commercial kit for the production of phage antibodies (Pharmacia) is already available.

Immunoblockade with human anti-CGRP MAbs offers the exciting possibility of probing the physiological role of CGRP directly in man. It is likely that antibody fragments will be used extensively in future because they may be readily expressed in active form from genes introduced into mammalian or bacterial cells (Hoogenboom *et al.,* 1991). Demonstration of an advantage of Fab' fragment in this project augurs well for its wider pharmacological application.

There seems to be no reason why anti-peptide MAbs or their fragments should not be investigated as therapeutic agents. The review of the pathophysiological roles of CGRP in Chapter 1 have suggested several therapeutic targets for CGRP blockade, including inflammation and migraine. Conversely, CGRP itself may be beneficial in conditions such as Raynaud's phenomenon and congestive cardiac failure. Since some anti-CGRP MAbs could potentiate the effect of CGRP (Shaw *et al.,* 1992), the binding of such a MAb to CGRP may yield a super-agonist complex with a prolonged duration of action.

Given evidence for CGRP receptor multiplicity and the wide range of biological effects attributed to CGRP, selective agonists or antagonists would appear

to be desirable for the treatment of particular conditions. However, selectivity of drug action is not always essential. In considering the potential value of immunoblockade as a therapeutic strategy, an analogy may be drawn between the use of an enzyme inhibitor and the use of immunoblockade. Pharmacologically, enzyme inhibition is relatively non-selective compared with receptor antagonism. Nevertheless, angiotensin converting enzyme and cyclooxygenase inhibitors, for example, have become established therapeutic agents while the therapeutic value of specific angiotensin II and prostaglandin receptor antagonists remains to be proven.

Immunoblockade with bispecific MAbs may be an interesting area for future research. Hybrid hybridomas may be produced by fusion of hybridomas of two different specificities (Milstein & Cuello, 1983). The resulting bispecific MAbs have two antigen binding sites, each with a different binding specificity. Alternatively, protein engineering techniques may be used to design dimeric antibody fragments or "diabodies" for bispecific interactions (Holliger *et al.*, 1993). The use of bispecific MAbs or diabodies offers the potential to block two mediators simultaneously. In conditions such as neurogenic inflammation, it is clear that CGRP is not the only mediator involved. Bispecific antibodies or antibody fragments with dual specificity for CGRP and substance P may be effective anti-inflammatory agents.

The therapeutic use of human MAbs and antibody fragments is limited by poor absorption, susceptibility to proteolysis and poor penetration of the blood-brain barrier. Advances in chemistry are offering solutions to these problems. Synthetic peptides derived from CDR sequences have properties similar to the intact antibody. Saragovi *et al.* (1991) determined the relevant contact residues and conformation involved in the binding of a MAb directed to the cell surface receptor of reovirus type 3, and developed a method for the organic synthesis of a non-peptide molecule that mimicked the binding and functional properties of the MAb. Thus CDRs of MAbs or antibody fragments from either hybridoma or phage antibody technologies may serve as templates for the design and synthesis of low molecular weight non-peptide drugs.

In conclusion, demonstration of the role of CGRP as a vasodilatory neurotransmitter by immunoblockade in this project has laid the foundation for further application of the technique to study the physiological and pathophysiological roles of CGRP. Exploitation of new technologies arising from molecular biology and chemistry should lead to the application of immunoblockade to study the physiological role of CGRP in man and, perhaps, the investigation of anti-CGRP antibodies or fragments as therapeutic candidates.

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