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.

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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

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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.

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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.

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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.

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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

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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,

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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.

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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.

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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

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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.

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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).

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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.

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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]-iodohistidyl^{10}-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

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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.

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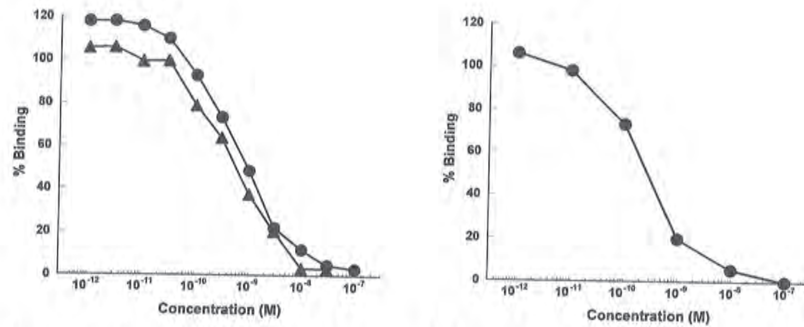

Figure 6.1: Displacement of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP binding to rat brain membrane preparation (a) or to SK-N-MC cell membrane preparation (b) by RαCGRP (circles) or HαCGRP (triangles). Values are the mean of 2 observations.

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

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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.

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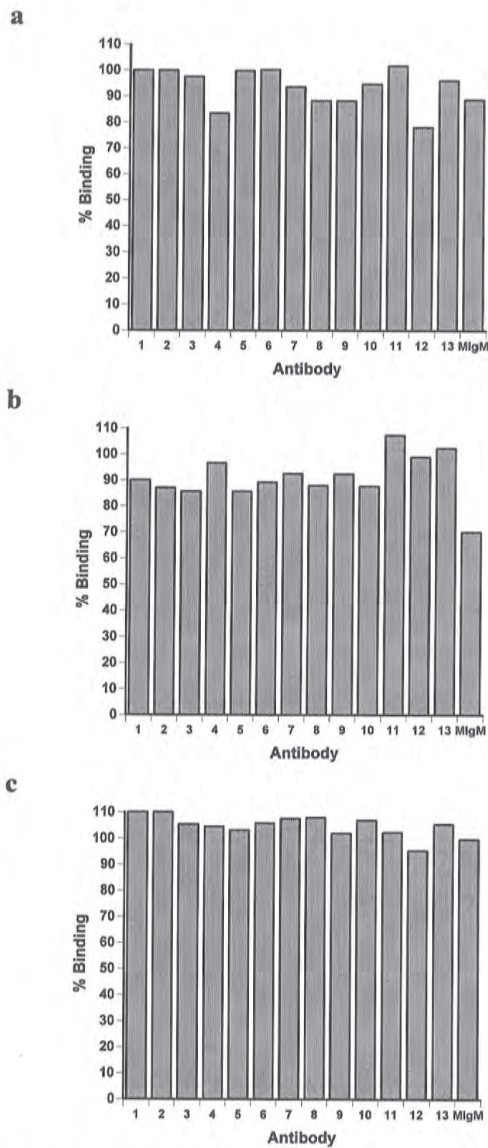

Figure 6.2: Effect of MAb Id1 to Id13 and non-specific mouse IgM (MIgM) on the binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to rat liver (a), rat brain (b) and human neuroblastoma SK-N-MC cell (c) membrane preparations. Specific binding is expressed as a percentage of vehicle control. Each value is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

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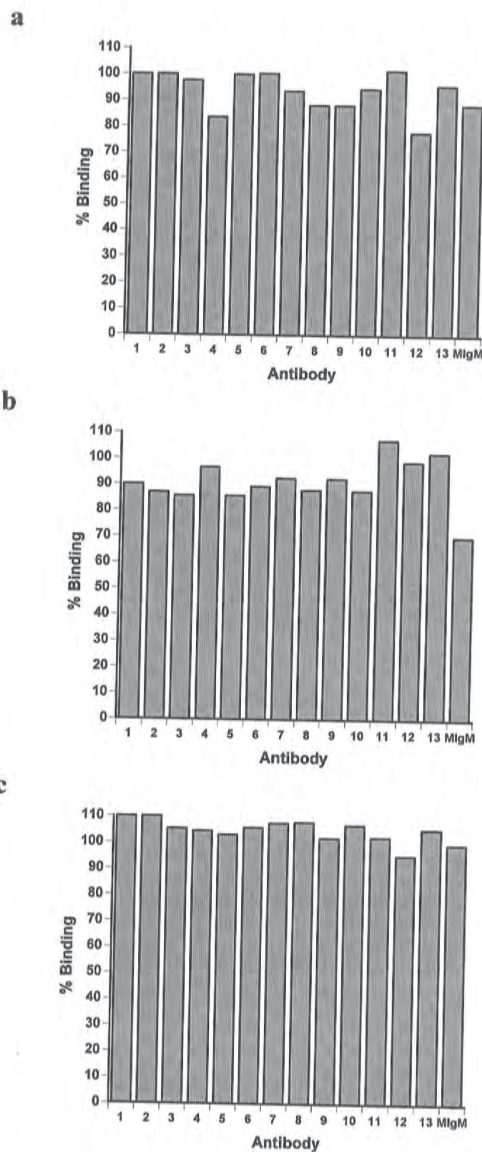

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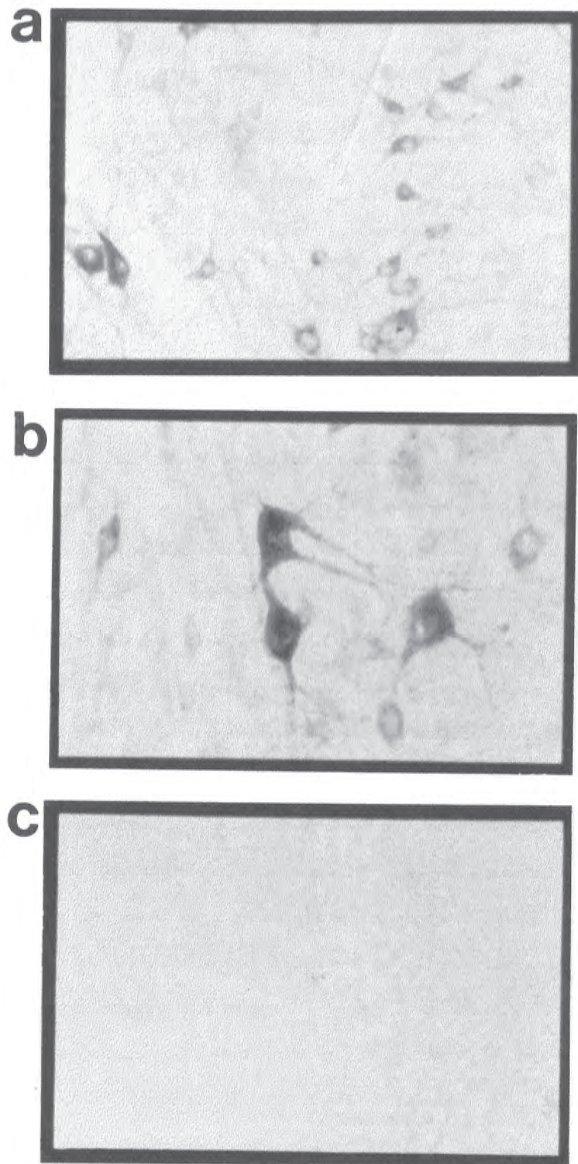

Figure 6.3: Immunostaining of cells by MAb Id1 (30 μg/ml) in the grey matter (a; ventral horn; x250) and white matter (b; x400) of the rat spinal cord. No staining was obtained by the use of a non-specific mouse IgM at 30 μg/ml (c; ventral horn; x250).

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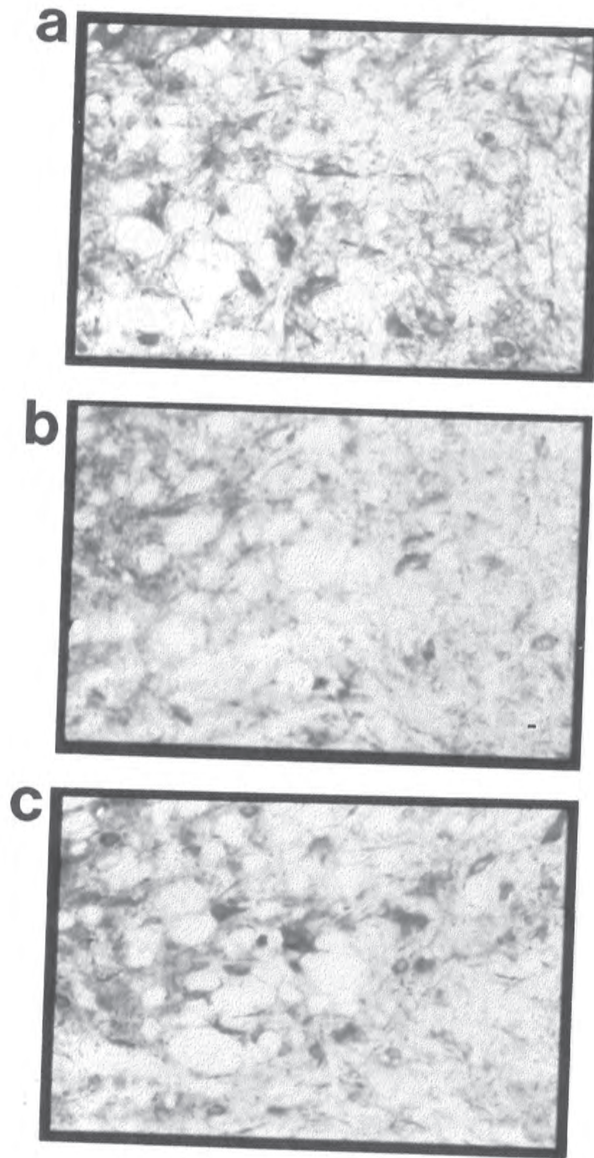

Figure 6.4: Immunostaining by MAb Id3 at 30 μg/ml (a; ventral horn; x250) was diminished by pre-incubation of the MAb with 800 μg/ml affinity-purified rabbit anti-CGRP PAbs (b) but not with 10 μM RαCGRP (c).

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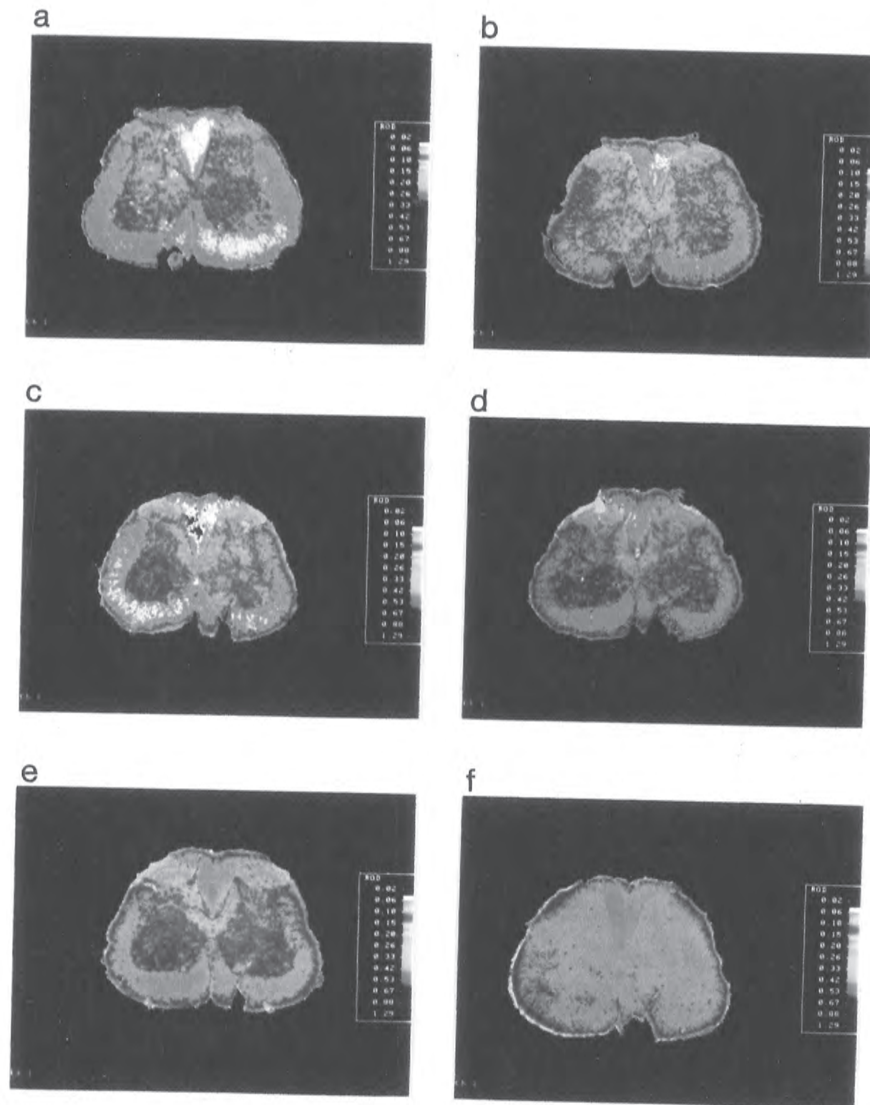

Figure 6.5: Image analysis of whole spinal cord sections immunostained with MAbs Id1 (a), Id5 (b), Id4 (c), Id11 (d), Id12 (e) and non-specific IgM (f). The colour coding for relative optical density is shown on the left of each figure. The concentration of MAbs used was 30 to 60 μg/ml.

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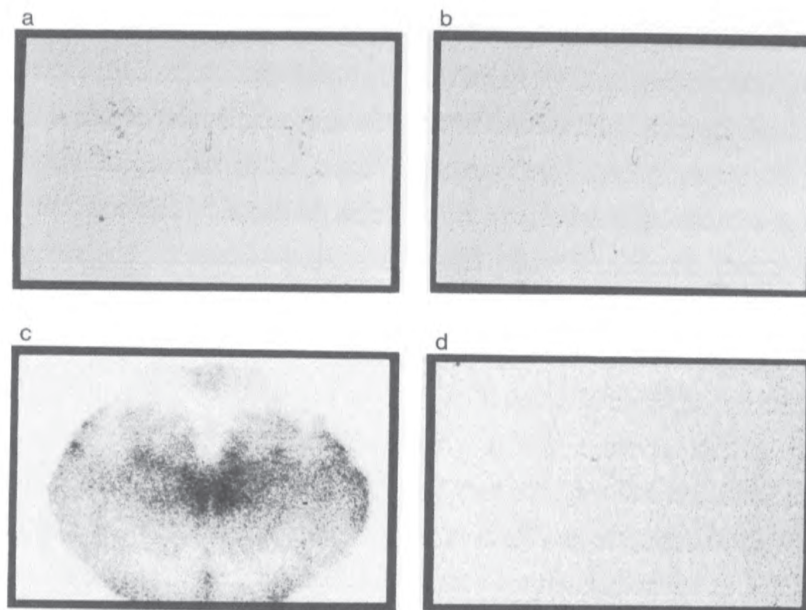

Figure 6.6: Spinal cord sections lightly stained with cresyl violet (a, b) and autoradiographs showing the binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to adjacent fresh (unfixed) spinal cord sections in the absence (c) or presence (d) of 10 μM RαCGRP.

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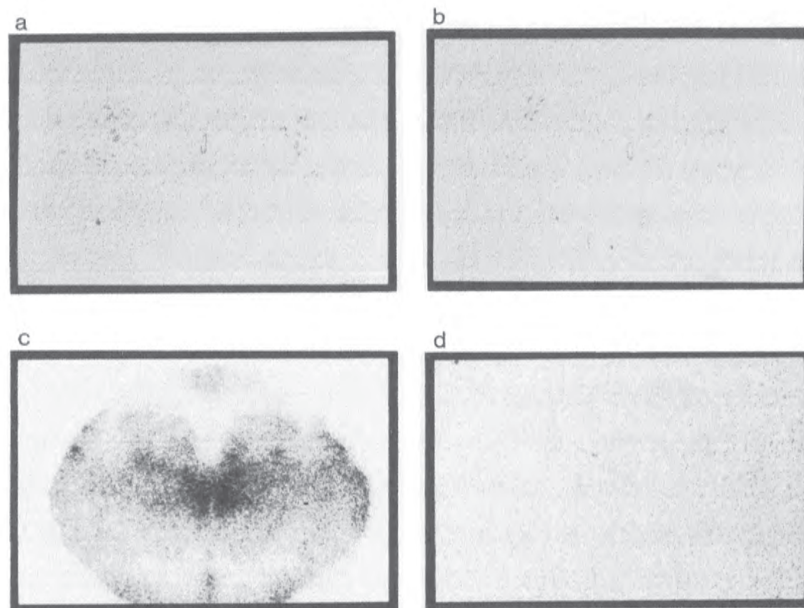

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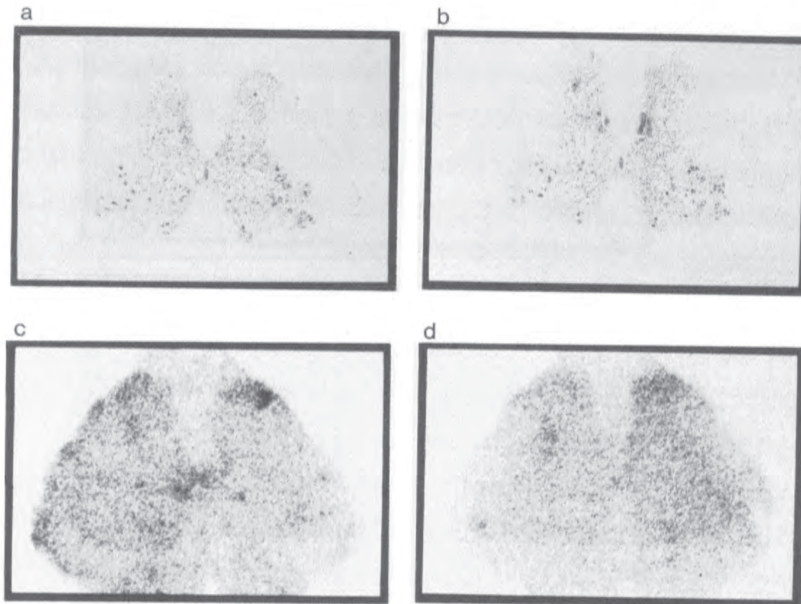

Figure 6.7: Cresyl violet stained spinal cord sections showing tissue morphology (a, b) and autoradiographs showing the binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to adjacent paraformaldehyde-fixed spinal cord sections in the absence (c) or presence (d) of 10 μM RαCGRP.

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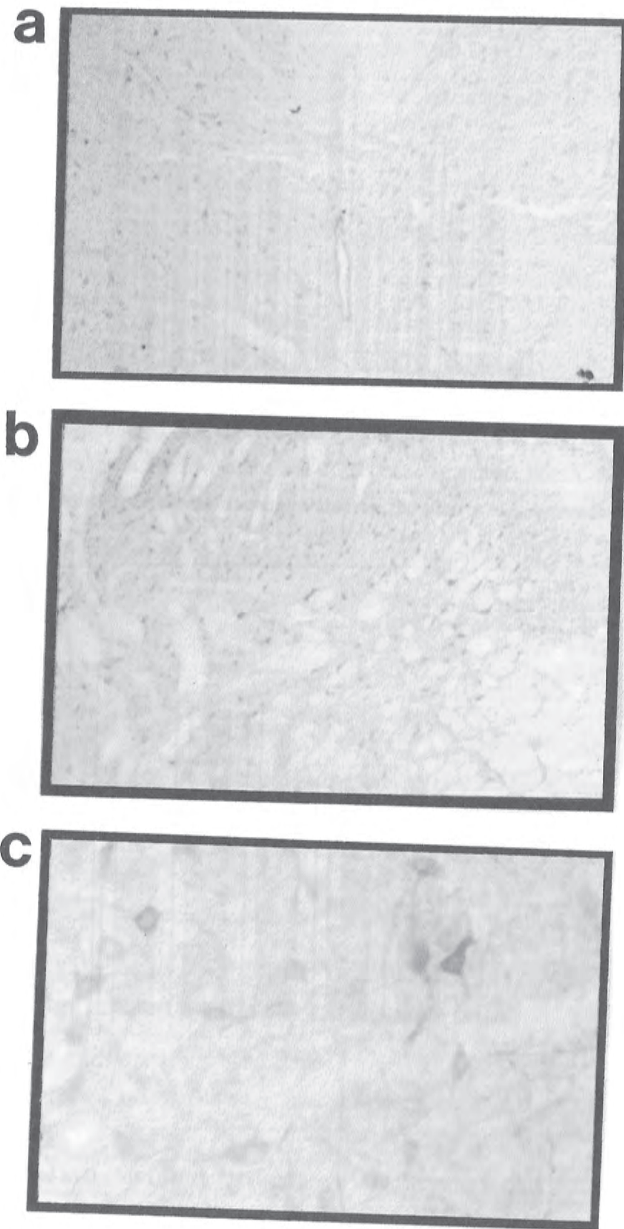

Figure 6.8: Immunostaining of fresh spinal cord sections with MAb Id1 at 15 μg/ml (a; central canal; x100), MAb Id3 at 30 μg/ml (b; dorsal horn; x100), and MAb Id12 at 60 μg/ml (c; dorsal horn; x400).

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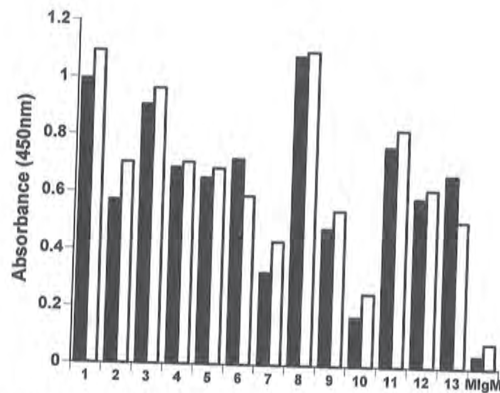

Figure 6.9: Effect of co-incubation with vehicle (filled bars) or RαCGRP (open bars) on the binding of Id MAbs (1 to 13) to rabbit affinity-purified anti-CGRP PAbs by indirect ELISA. Non-specific mouse IgM (10 μg/ml; MIgM) was used as a control. Values are the mean of two observations.

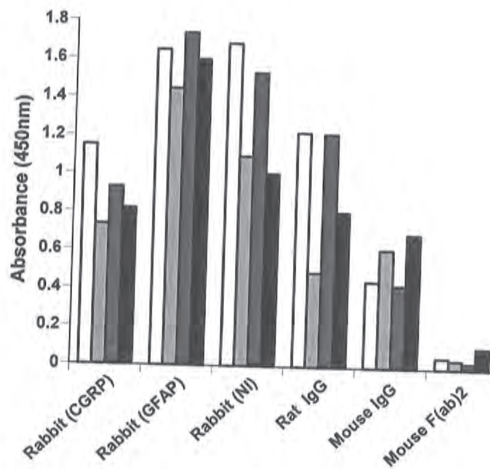

Figure 6.10: Binding of MAbs Id1 (white), Id2 (light grey), Id3 (dark grey) and Id4 (black) to rabbit anti-CGRP, anti-glial fibrillary acidic protein (GFAP), and non-immune (NI) IgG, normal rat IgG, mouse MAb R1.50 IgG and MAb R1.50 $F(ab')_2$ fragments by indirect ELISA. Similar binding was observed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

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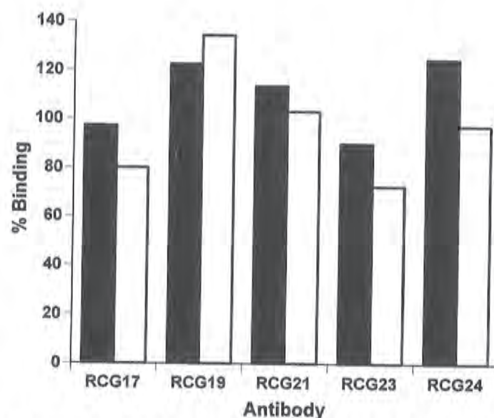

Figure 6.11: Effect of RCG MAbs on the binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to human neuroblastoma SK-N-MC cell (filled bars) or rat brain (open bars) membrane preparations. Binding is expressed as a percentage of specific binding in the presence of cell culture medium (10FD) alone. The final 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

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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

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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

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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

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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

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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 $H\alpha CGRP_{8-37}$ as a CGRP receptor antagonist.

7.2. Methods

Materials	**Supplier**
HαCGRP	Peninsula
HβCGRP	Peninsula
$H\alpha CGRP_{8-37}$	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

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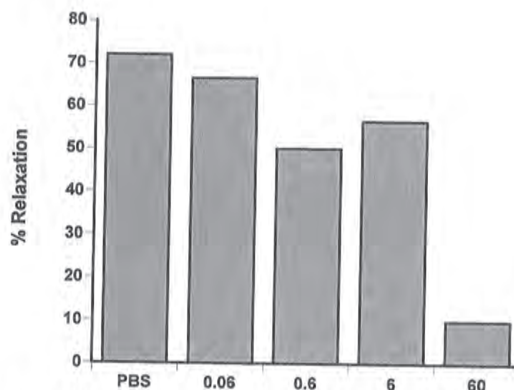

Figure 7.1: Effect of MAb R1.50 concentration on the relaxation of porcine coronary artery segments by 50 nM HβCGRP. Coronary artery segments were pre-contracted with 25 mM KCl and pre-incubated with MAb R1.50 for 60 minutes prior to exposure to HβCGRP. Control tissues were pre-incubated with PBS vehicle 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 $H\alpha CGRP_{8-37}$ as an antagonist of HαCGRP was tested at concentrations of 1 μM and 5 μM. $H\alpha CGRP_{8-37}$ was added to tissue baths 30 minutes prior to the start of CGRP concentration-response studies.

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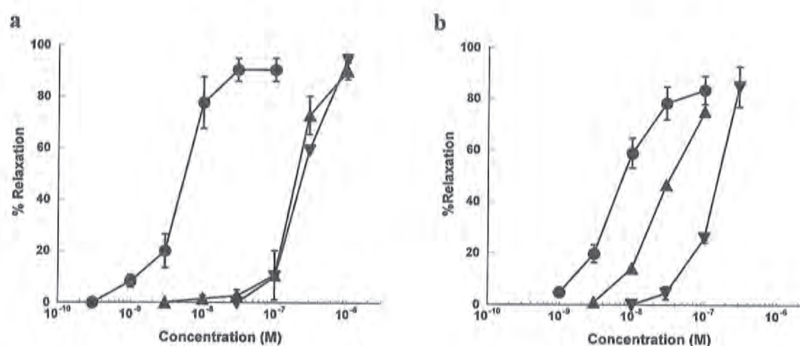

Figure 7.2: Effect of MAb R1.50 IgG at 12 μg/ml (triangles) or 60 μg/ml (inverted triangles) on the relaxation of KCl pre-contracted coronary artery segments by HαCGRP (a) and HβCGRP (b). Control concentration-response relationships were obtained in the presence of PBS vehicle. Mean values and standard 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 $H\alpha CGRP_{8\text{-}37}$ (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 $H\alpha CGRP_{8\text{-}37}$ 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.

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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).

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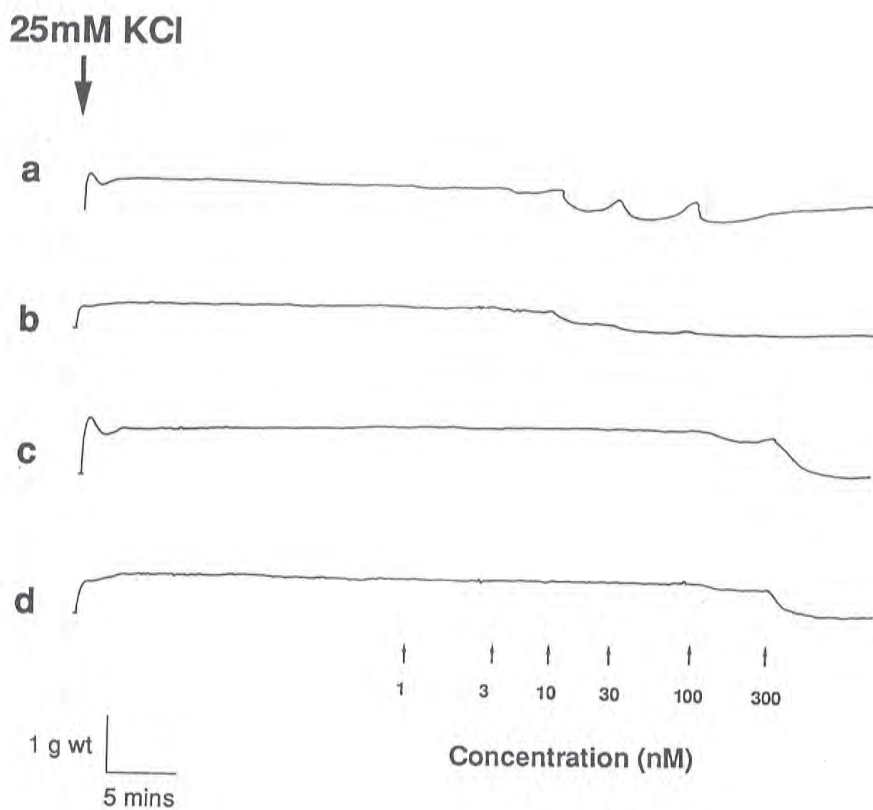

Figure 7.3: Original traces showing the relaxation of KCl pre-contracted coronary artery segments by HβCGRP in the presence of PBS vehicle (a,b) or 60 μg/ml MAb R1.50 IgG (c,d). MAb R1.50 or vehicle was added to tissue baths 60 minutes prior to the start of the CGRP concentration-response study.

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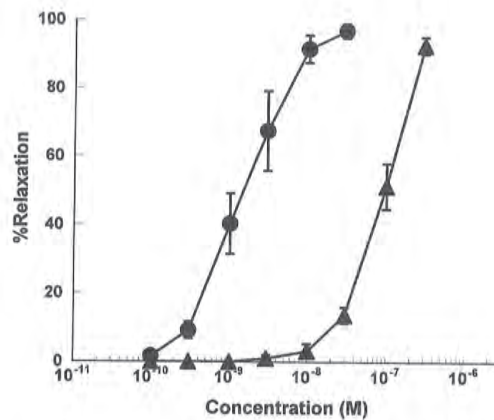

Figure 7.4: Effect of MAb R1.50 Fab' fragments (triangles) or PBS vehicle (circles) on the relaxation of KCl pre-contracted porcine coronary artery segments by HαCGRP. Tissues were incubated with the Fab' fragment or PBS vehicle for 30 minutes before the start of the CGRP concentration-response study.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

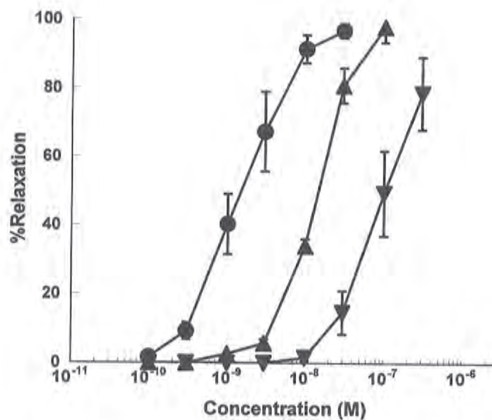

Figure 7.5: Effect of HαCGRP$_{8\text{-}37}$ at 1 μM (triangles) or 5 μM (inverted triangles) on the relaxation of KCl pre-contracted porcine coronary artery segments by HαCGRP compared with control (PBS; circles). HαCGRP$_{8\text{-}37}$ or PBS was added to tissue baths 60 minutes before the start of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

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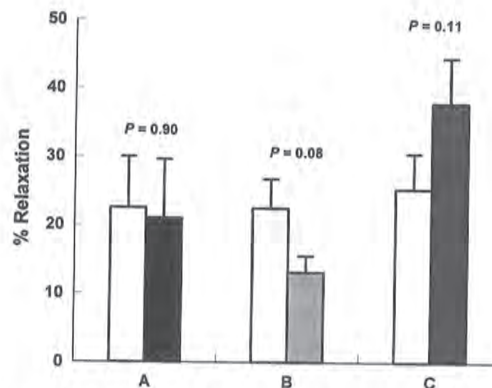

Figure 7.6: Effect of 60 μg/ml MAb R1.50 IgG (A; black bar), 60 μg/ml MAb R1.50 Fab' fragment (B; light grey bars) or 5 μM HαCGRP$_{8-37}$ (C; dark grey bar) on capsaicin-induced relaxation of KCl pre-contracted coronary artery segments compared with concurrent vehicle controls (A, B, and C; white bars). 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

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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,

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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

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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)

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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.).

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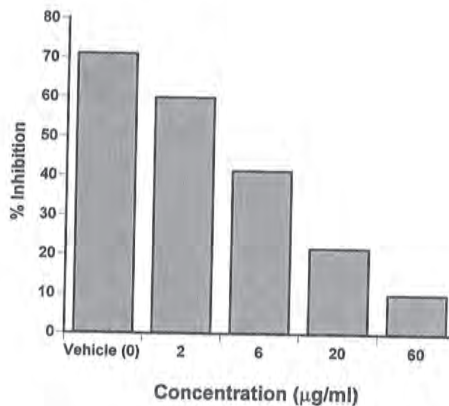

Figure 8.1: Effect of MAb C4.19 at various protein concentrations on the inhibitory effect of 10 nM RαCGRP. The effect of CGRP was expressed as % inhibition relative to stable pre-dosing contractions. Mean responses from 2 experiments are shown.

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

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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.

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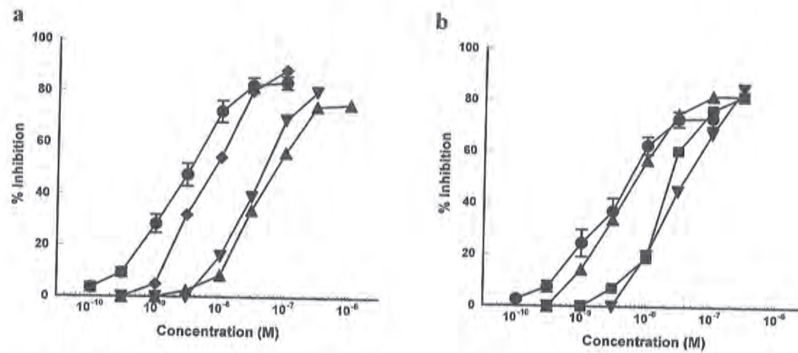

Figure 8.2: Concentration-response curves observed for RαCGRP (a) or RβCGRP (b) in the presence of PBS vehicle (circles) or purified MAbs R2.73 (triangles), C4.6 (inverted triangles), P3.71 (diamonds) and P3.77 (squares) at 60 μg/ml protein. The effect of CGRP was expressed as % inhibition relative to 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).

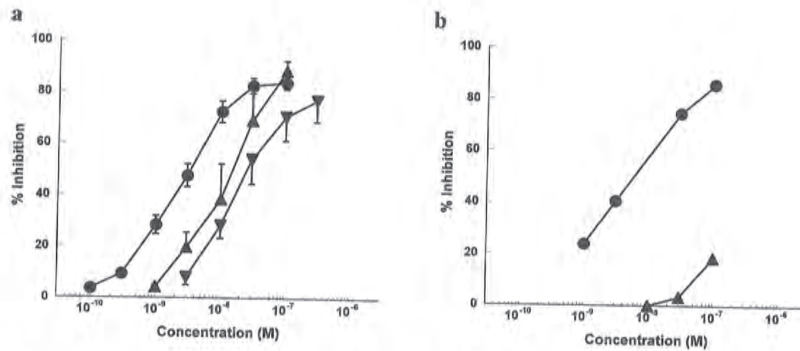

Figure 8.3: Concentration-response curves observed for RαCGRP (a) and HαCGRP (b) in the presence of PBS vehicle (circles) or purified MAb R1.50 at a protein concentration of 60 μg/ml (triangles) or 600 μg/ml (inverted triangle). The effect of CGRP was expressed as % inhibition relative to stable pre-dosing 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.

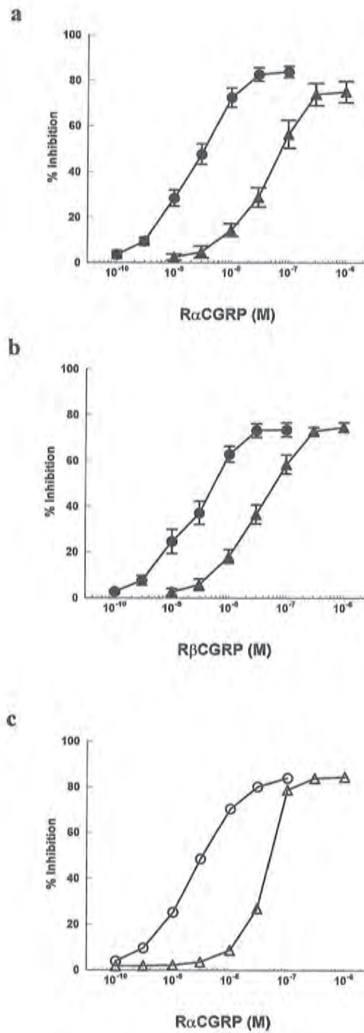

Figure 8.4: Concentration-response curves observed for RαCGRP (a) or RβCGRP (b) in the presence of PBS vehicle (closed circles) or purified MAb C4.19 ascites at 60 μg/ml protein (closed triangles). The effect of CGRP was expressed as % inhibition relative to stable pre-dosing contractions. Mean responses (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.

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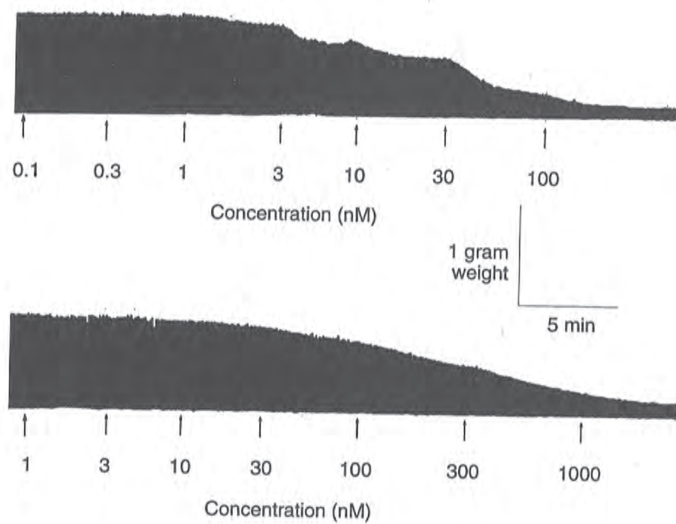

Figure 8.5: Original traces showing the inhibitory effect of RαCGRP on electrically-stimulated contractions of the isolated rat vas deferens in the presence of irrelevant control MAb MAR 18.5 (top) or MAb C4.19 at 60 μg/ml protein (bottom). The traces were obtained from paired tissues from a single animal.

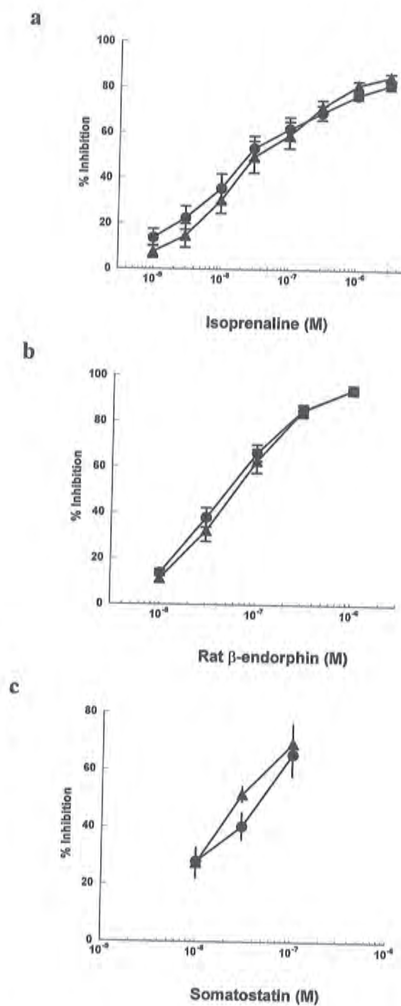

Figure 8.6: Concentration-response curves observed for isoprenaline (a), rat β-endorphin (b) and somatostatin (c) in the presence of PBS vehicle (circles) or MAb C4.19 at 60 μg/ml protein (triangles). The effects of drugs were expressed as % inhibition relative to stable pre-dosing contractions. Mean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

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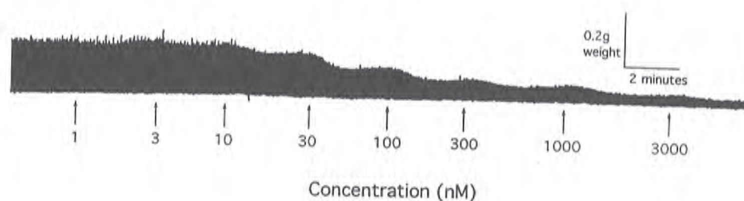

Figure 8.7: Original traces showing the inhibitory effect of isoprenaline on electrically-stimulated contractions of the isolated rat vas deferens. Cumulative concentrations of isoprenaline are shown.

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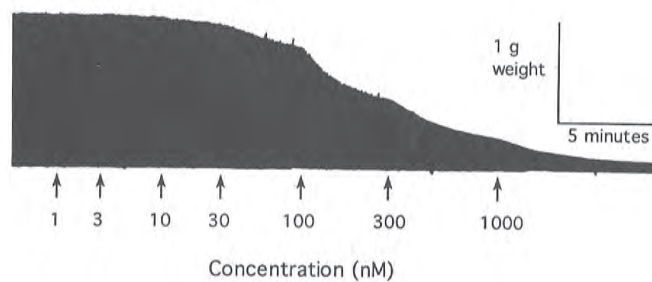

Figure 8.8: Original traces showing the inhibitory effect of rat β-endorphin on electrically-stimulated contractions of the isolated rat vas deferens. Cumulative concentrations of rat β-endorphin are shown.

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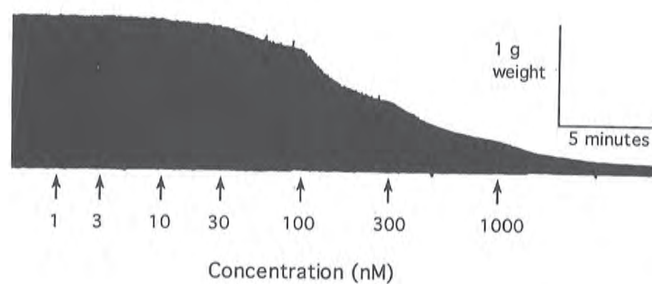

Figure 8.8: Original traces showing the inhibitory effect of rat β-endorphin on electrically-stimulated contractions of the isolated rat vas deferens. Cumulative concentrations of rat β-endorphin are shown.

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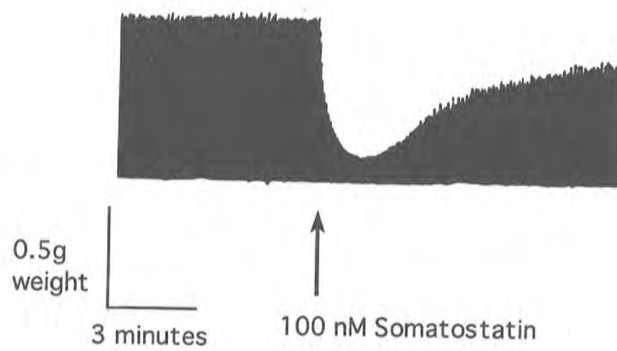

Figure 8.9: Original traces showing the inhibitory effect of somatostatin on electrically-stimulated contractions of the isolated rat vas deferens. Individual tissues were exposed to a single concentration because tachyphylaxis develops to the effect of somatostatin.

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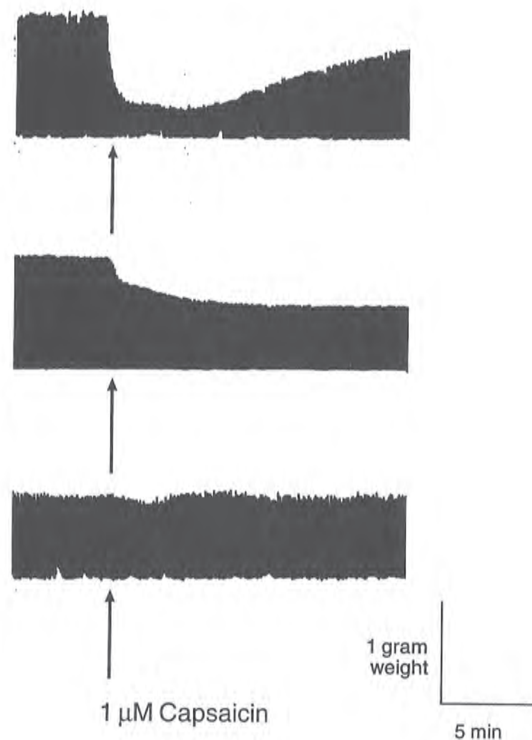

Figure 8.10: Original traces showing the inhibitory effect of 1 μM capsaicin on electrically-stimulated contractions of the isolated rat vas deferens in the presence of irrelevant control MAb directed against thyroid-stimulating hormone (top) or MAb C4.19 at 60 μg/ml protein (middle and bottom). The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

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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

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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

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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

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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). HαCGRP$_{8\text{-}37}$ 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$_{8\text{-}37}$ 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$_{8\text{-}37}$ (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$_{8\text{-}37}$. Thus endogenous CGRP appears to be a major neurotransmitter that mediates neurogenic vasodilatation following spinal cord stimulation in the rat. HαCGRP$_{8\text{-}37}$ 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$_{8\text{-}37}$ (Delay-Goyet *et al.*, 1992; Escott

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& 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

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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.

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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$_{8-37}$.

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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;

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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:

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

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

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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 Hα$CGRP_{8-37}$

Vehicle or Hα$CGRP_{8-37}$ (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 Hα$CGRP_{8-37}$ and RP-67,580

The neurokinin$_1$ (NK_1) antagonist RP-67,580 (10 mg/kg) and Hα$CGRP_{8-37}$ (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.

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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.

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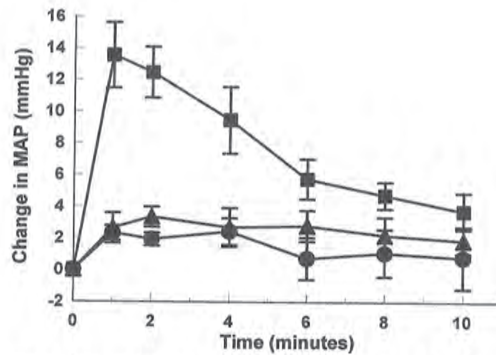

Figure 9.1: Effect of 1 mg/rat (circles) or 3 mg/rat (squares) i.v. MAb C4.19 IgG and $H\alpha CGRP_{8-37}$ (100 nmol/kg i.v.; triangles) on baseline MAP. Mean results are plotted with standard error bars (n=4 to 6)

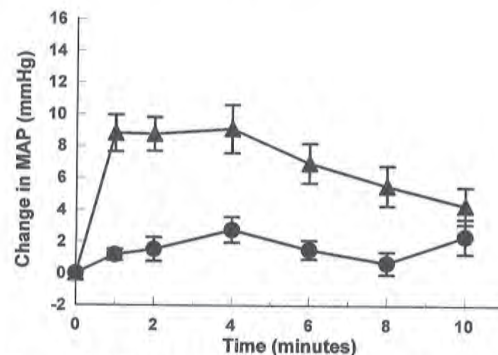

Figure 9.2: Effect of MAb C4.19 Fab' fragment (2 mg/rat i.v.; triangles) and normal mouse Fab' fragment (2 mg/rat i.v.; circles) on baseline MAP. Mean results are plotted with standard error bars (n=6)

9.3. Results

9.3.1 Effects on baseline MAP

$H\alpha CGRP_{8-37}$ (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)

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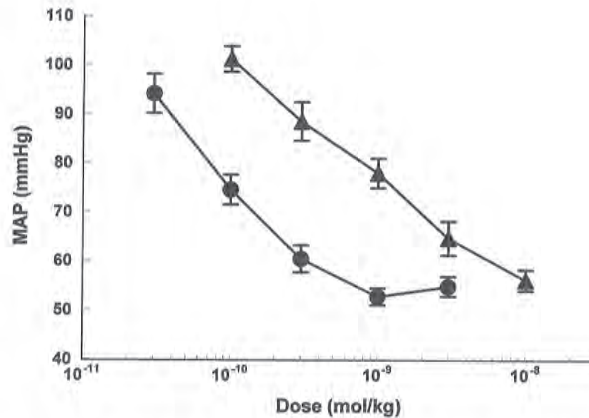

Figure 9.3: Effect of 1 mg/rat i.v. MAb C4.19 IgG (triangles) or 1 mg/rat i.v. control MAb IgG (circles) on the dose-response relationship for the effect of i.v. RαCGRP on MAP. Control MAbs were anti-TSH MAb or mouse anti-rat MAb MAR 18.5. The baseline MAP was not significantly different between the 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).

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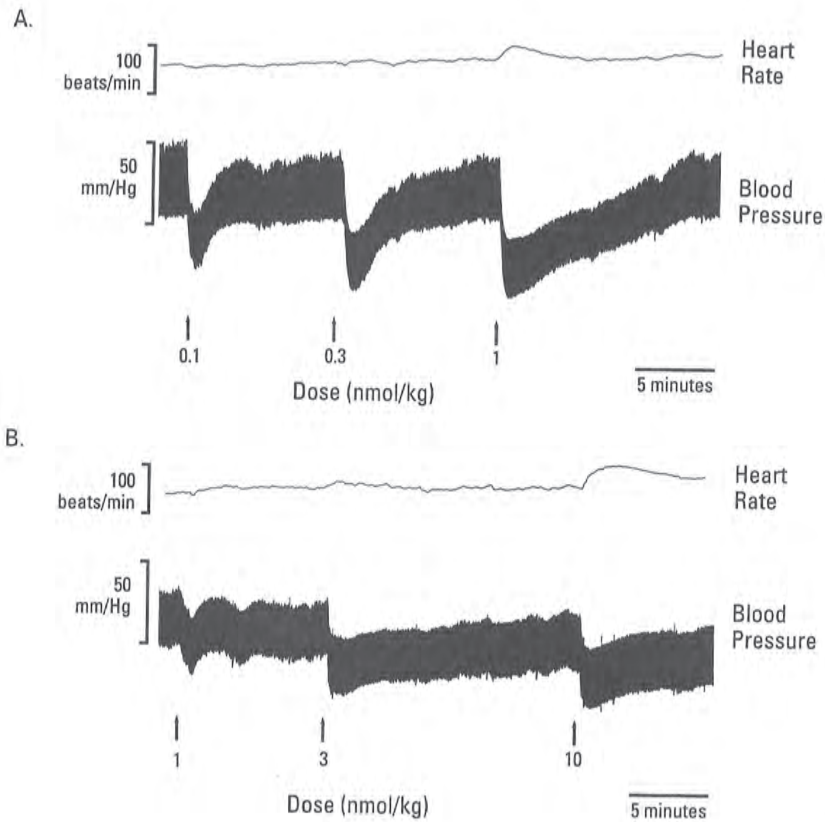

Figure 9.4: Original recordings of blood pressure and heart rate in a pentobarbitone-anaesthetized rat showing the effect of i.v. doses of RαCGRP following pre-treatment with 1 mg/rat irrelevant anti-TSH MAb IgG (a) or 1 mg/rat MAb C4.19 IgG (b). MAbs were given 60 minutes before the start of RαCGRP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

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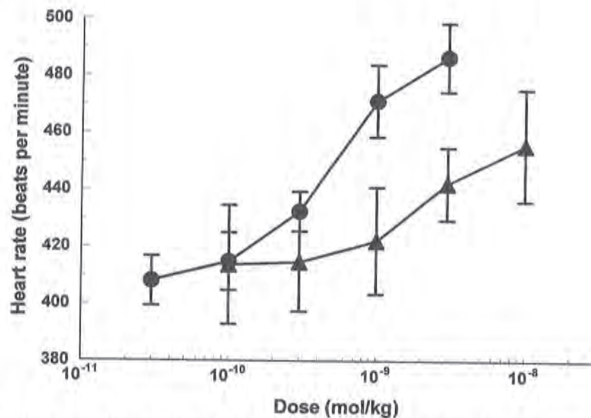

Figure 9.5: Effect of 1 mg/rat i.v. MAb C4.19 IgG (triangles) and 1 mg/rat i.v. control MAb IgG (circles) on the dose-response relationship for the effect of RαCGRP on heart rate. Control MAbs were anti-TSH MAb or mouse anti-rat MAb MAR 18.5. Mean results are plotted with standard error bars (n=6)

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).

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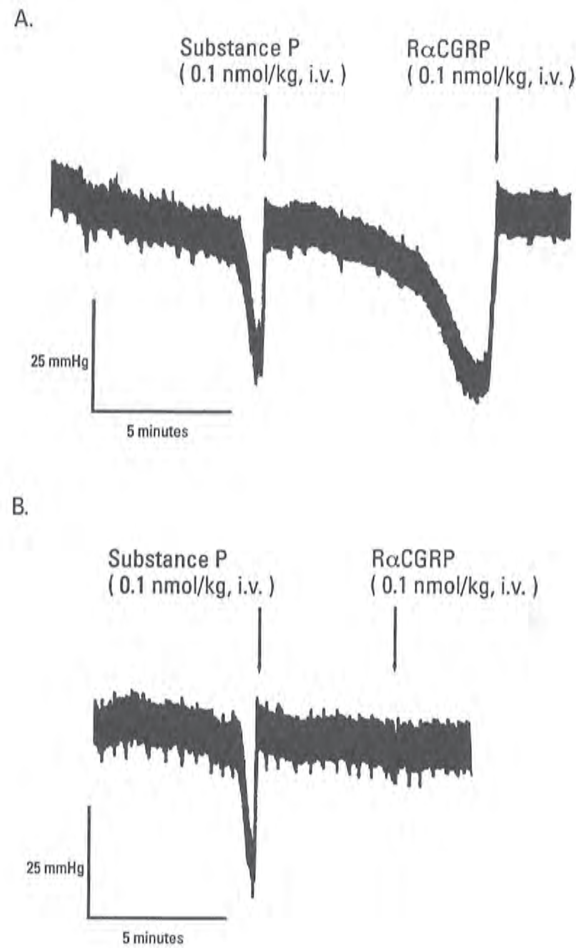

Figure 9.6: Original blood pressure recording showing control responses to 0.1 nmol/kg i.v. RαCGRP and 0.1 nmol/kg i.v. substance P (A). The response to RαCGRP was completely blocked but the response to substance P was unaltered 30 minutes following i.v. injection of 1 mg/rat MAb C4.19 IgG (B).

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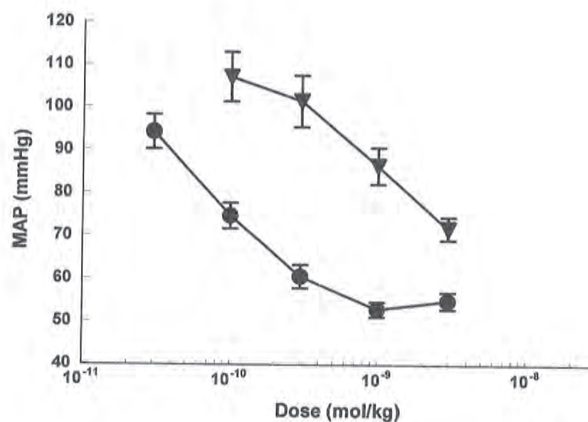

Figure 9.7: Effect of 2 mg/rat i.v. MAb C4.19 Fab' fragment (triangles) on the dose-response relationship for the effect of i.v. RαCGRP on MAP. The dose-response relationship of RαCGRP following treatment with 1mg/rat control MAb IgG (circles) is plotted for comparison. Mean results are plotted with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

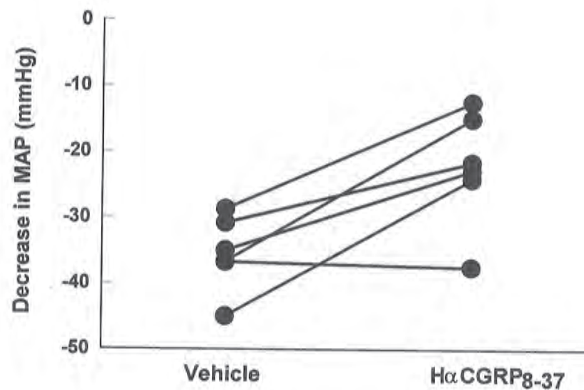

Figure 9.8: Effect of 100 nmol/kg i.v. HαCGRP$_{8\text{-}37}$ on the hypotensive response to 0.1 nmol/kg i.v. RαCGRP in 6 rats. HαCGRP$_{8\text{-}37}$ was given 3 minutes before RαCGRP.

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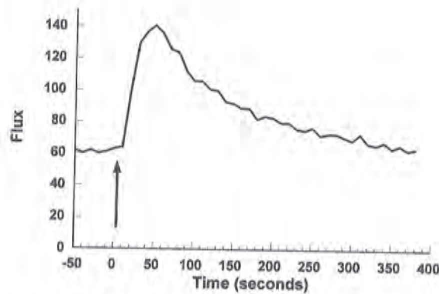

Figure 9.9: Typical change in skin blood flow following antidromic stimulation of the saphenous nerve (indicated by arrow) in a rat. Simulation parameters were 2 Hz, 10 V, 1 millisecond, for 10 seconds.

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

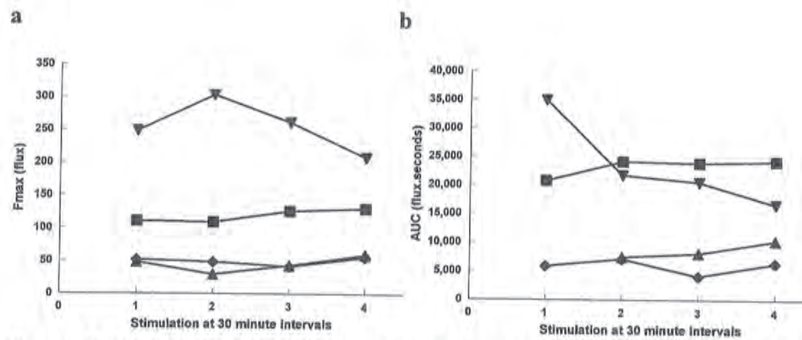

Figure 9.10: Fmax (a) and AUC (b) following repeated stimulation of the saphenous nerve in individual rats at 30 minutes intervals.

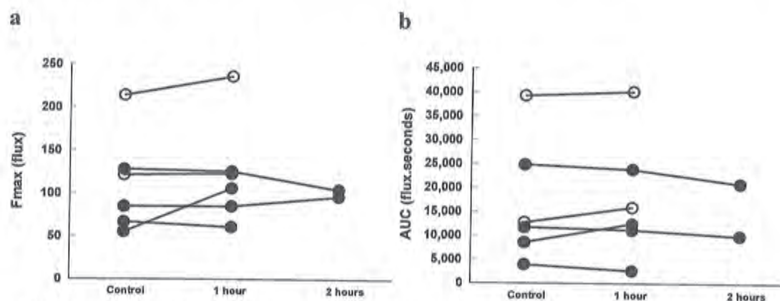

Figure 9.11: Effect of MAb C4.19 IgG at 1 mg/rat i.v. (open circles) or 3 mg/rat i.v. (closed circles) on Fmax (a) and AUC (b) in individual rats. Antidromic nerve stimulation was performed 1 or 2 hours following treatment.

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).

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a

b

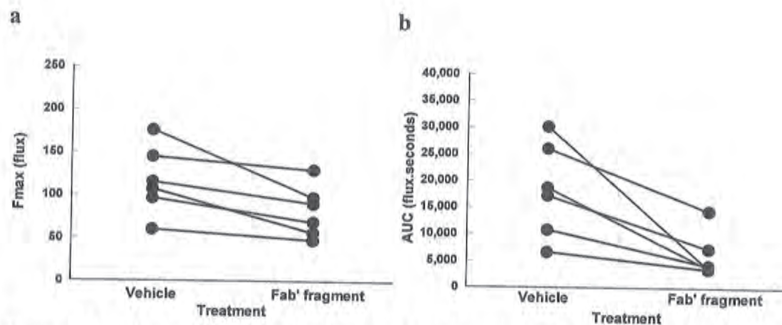

Figure 9.12: Effect of 2 mg/rat i.v. MAb C4.19 Fab' fragment on Fmax (a) and AUC (b) in individual rats. Antidromic nerve stimulation was performed 30 minutes following treatment.

a

b

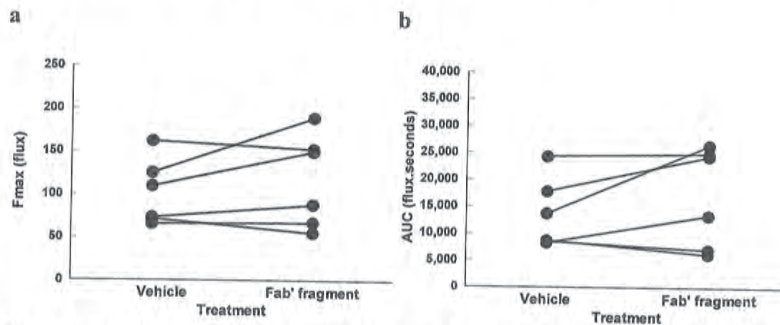

Figure 9.13: Effect of 2 mg/rat i.v. normal mouse Fab' fragment on Fmax (a) and AUC (b) in individual rats. Antidromic nerve stimulation was performed 30 minutes following treatment.

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]).

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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.

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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
$H\alpha CGRP_{8-37}$	-74.6 (-56.2 to -92.9)	0.04
$H\alpha CGRP_{8-37}$ 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 $H\alpha CGRP_{8-37}$

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 $H\alpha CGRP_{8-37}$ (P=0.30 and 0.27 respectively).

9.3.3.8. Co-administration of $H\alpha CGRP_{8-37}$ 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 $H\alpha CGRP_{8-37}$ alone. However, the percentage decrease in AUC with two antagonists was not greater than that observed with $H\alpha CGRP_{8-37}$ 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.

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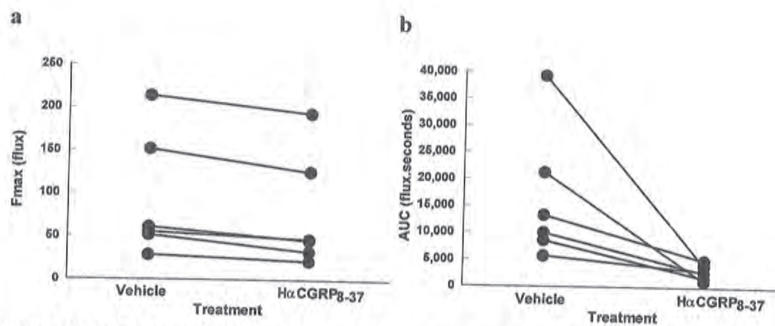

Figure 9.14: Effect of 100 nmol/kg i.v. HαCGRP$_{8-37}$ on Fmax (a) and AUC (b) in individual rats. HαCGRP$_{8-37}$ was given 3 minutes before antidromic nerve stimulation.

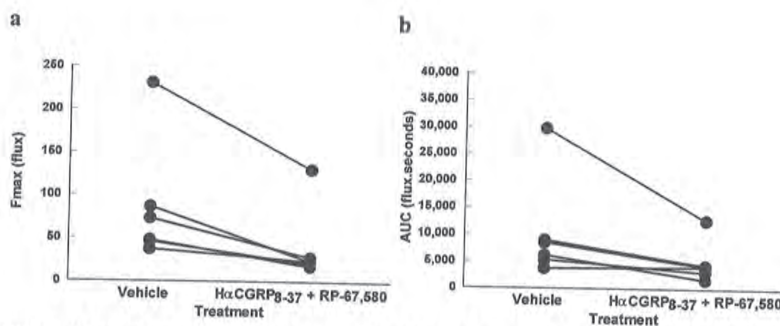

Figure 9.15: Effect of co-administration of 100 nmol/kg i.v. HαCGRP$_{8-37}$ and 10 mg/kg i.v. RP-67,580 on Fmax (a) and AUC (b) in individual rats. HαCGRP$_{8-37}$ and RP-67,580 were given 3 minutes and 15 minutes before antidromic nerve stimulation respectively.

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

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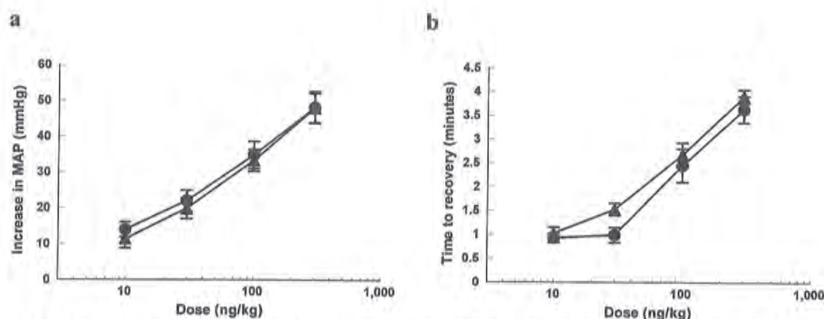

Figure 9.16: Effect of 2 mg/rat i.v. MAb C4.19 Fab' fragment (triangles) or vehicle (circles) on pressor response to i.v. angiotensin II (a) and time to recovery of MAP following pressor response to angiotensin II (b). Mean results are plotted with standard error bars (n=6).

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$_{8\text{-}37}$.

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

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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

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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

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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

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(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 $H\alpha CGRP_{8-37}$ 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

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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 $H\alpha CGRP_{8-37}$. 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 $H\alpha CGRP_{8-37}$ produced vasoconstriction in the isolated rat mesenteric vascular bed. $H\alpha CGRP_{8-37}$ 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 $H\alpha CGRP_{8-37}$ (300 nmol/kg/min) but this was accompanied by tachycardia. An increase in MAP following infusion of a lower dose of $H\alpha CGRP_{8-37}$ (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 $H\alpha CGRP_{8-37}$. 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.

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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

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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.

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

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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.

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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

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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

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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.

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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.

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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

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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

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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.

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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

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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

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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.

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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

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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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