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CL/RAMP2 and CL/RAMP3 produce pharmacologically distinct adrenomedullin receptors: a comparison of effects of adrenomedullin$_{22-52}$, CGRP$_{8-37}$ and BIBN4096BS

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1 Adrenomedullin (AM) has two known receptors formed by the calcitonin receptor-like receptor (CL) and receptor activity-modifying protein (RAMP) 2 or 3: We report the effects of the antagonist fragments of human AM and CGRP (AM$_{22-52}$ and CGRP$_{8-37}$) in inhibiting AM at human (h), rat (r) and mixed species CL/RAMP2 and CL/RAMP3 receptors transiently expressed in Cos 7 cells or endogenously expressed as rCL/rRAMP2 complexes by Rat 2 and L6 cells.

2 AM$_{22-52}$ (10 μM) antagonised AM at all CL/RAMP2 complexes (apparent pA_2 values: 7.34$\pm$0.14 (hCL/hRAMP2), 7.28$\pm$0.06 (Rat 2), 7.00$\pm$0.05 (L6), 6.25$\pm$0.17 (rCL/hRAMP2)). CGRP$_{8-37}$ (10 μM) resembled AM$_{22-52}$ except on the rCL/hRAMP2 complex, where it did not antagonise AM (apparent pA_2 values: 7.04$\pm$0.13 (hCL/hRAMP2), 6.72$\pm$0.06 (Rat2), 7.03$\pm$0.12 (L6)).

3 On CL/RAMP3 receptors, 10 μM CGRP$_{8-37}$ was an effective antagonist at all combinations (apparent pA_2 values: 6.96$\pm$0.08 (hCL/hRAMP3), 6.18$\pm$0.18 (rCL/rRAMP3), 6.48$\pm$0.20 (rCL/hRAMP3)). However, 10 μM AM$_{22-52}$ only antagonised AM at the hCL/hRAMP3 receptor (apparent pA_2 6.73$\pm$0.14).

4 BIBN4096BS (10 μM) did not antagonise AM at any of the receptors.

5 Where investigated (all-rat and rat/human combinations), the agonist potency order on the CL/RAMP3 receptor was AM $\sim$ βCGRP $>$ αCGRP.

6 rRAMP3 showed three apparent polymorphisms, none of which altered its coding sequence.

7 This study shows that on CL/RAMP complexes, AM$_{22-52}$ has significant selectivity for the CL/RAMP2 combination over the CL/RAMP3 combination. On the mixed species receptor, CGRP$_{8-37}$ showed the opposite selectivity. Thus, depending on the species, it is possible to discriminate pharmacologically between CL/RAMP2 and CL/RAMP3 AM receptors.
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Keywords: CGRP; CGRP$_{8-37}$; adrenomedullin; adrenomedullin$_{22-52}$; calcitonin receptor-like receptor; CL; RAMP2; RAMP3

Abbreviations: AM, adrenomedullin; BIBN4096BS, 1-piperidinecarboxamide, *N*-[2-[[5amino-1-[[4-(4-pyridinyl)-1-piperazinyl]-carbonyl]pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2*H*)-quinazolinyl); CGRP, calcitonin gene-related peptide; CL, calcitonin receptor-like receptor; [cys(ACM)^{2,7}]αCGRP, Cys-acetoamidomethyl^{2,7} human αCGRP; [cys(Et)^{2,7}]αCGRP, Cys-ethylamide^{2,7} human αCGRP; r, rat; RAMP, receptor activity-modifying protein

Introduction

AM is an essential vascular peptide; its presence in the developing foetus governs the formation of intact vasculature and thus foetal survival (Caron & Smithies, 2001; Shindo *et al*., 2001). These studies support the well-recognised role of AM in cell growth, and further the concept that AM may be involved in angiogenesis (Miller *et al*., 1996; Withers *et al*., 1996; Nikitenko *et al*., 2002). The pharmacology of receptors responsive to AM has been examined in many tissues and cell lines (see Hinson *et al*., 2000; Hay & Smith, 2001 for reviews). Specific AM receptors can be characterised by high affinity for AM and $\geqslant$100-fold lower affinity for the other members of the calcitonin family of peptides (Coppock *et al*., 1999). The effects of AM at such receptors can be inhibited by the AM antagonist fragment AM$_{22-52}$ (Eguchi *et al*., 1994). The αCGRP antagonist fragment CGRP$_{8-37}$ and the amylin receptor antagonist AC187 can also antagonise specific AM receptors, but only at high concentrations ($>$5 μM) (Coppock *et al*., 1999). AM also activates CGRP receptors, and these effects can be inhibited by CGRP$_{8-37}$ (Nagoshi *et al*., 2002). However, this description is likely to be an oversimplification. AM$_{22-52}$ is the only antagonist available which is specific for AM receptors, and this is a relatively low-affinity peptide. Without better antagonists, it is difficult to separate receptor

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subtypes which may exist in tissues that are likely to contain very complex mixtures of receptors, for example, vas deferens (Poyner *et al*., 1999; Wu *et al*., 2000).

Generally, it seems that AM_{22-52} antagonises the effects of AM but not CGRP. However, high concentrations of AM_{22-52} are often required due to the low affinity of this antagonist, and observations of unusual pharmacology, potentially attributable to the existence of subtypes of AM receptors, have been noted. In the rat vas deferens, the effects of AM or [Cys(Et)^{2,7}]αCGRP (a putative '$CGRP_2$'-receptor selective agonist; Dumont *et al*., 1997) were more potently antagonised by BIBN4096BS than those of either α or βCGRP (Wu *et al*., 2000). In the hind limb vascular bed of the cat, AM_{22-52} could not antagonise the effects of AM, but could inhibit the effects of CGRP (Champion *et al*., 1997). In this system, $CGRP_{8-37}$ could inhibit responses to CGRP but not AM.

Two AM receptor subtypes have now been defined in molecular terms: AM_1, composed of CL with RAMP2, and AM_2, composed of CL and RAMP3 (McLatchie *et al*., 1998; Poyner *et al*., 2002). RAMP2 and RAMP3 can be differentially regulated in *in vivo* models of the disease (Ono *et al*., 2000). For example, in a rat model of obstructive neuropathy, CL, RAMP1 and RAMP2 mRNA levels were upregulated, but RAMP3 levels were unchanged (Nagae *et al*., 2000). At present, there is no pharmacological separation of AM_1 and AM_2 receptors although it has been reported that a mouse RAMP3/rat CL (rCL) complex is more sensitive to the effects of CGRP than its RAMP2 counterpart (Husmann *et al*., 2000). The mouse RAMP3/rat CL receptor is considered a mixed AM/CGRP receptor, but in terms of the effects of antagonists, specific AM_1 and AM_2 receptors have never been thoroughly characterised. Recent studies have examined the effects of $CGRP_{8-37}$ and AM_{22-52} at human, bovine and porcine CL complexes with RAMPs1–3 (Aiyar *et al*., 2001; 2002). However, these studies were performed in HEK293 cells which are known to express endogenous RAMPs (particularly RAMP2) and/or CL (Aiyar *et al*., 1996; Kuwasako *et al*., 2001). Most other analyses have been based on binding studies. Therefore, the functional effects of these antagonists at exclusive CL/RAMP3 complexes have never been examined. Furthermore, although CL has been cloned from several species (Elshourbagy *et al*., 1998; Aiyar *et al*., 2001; 2002), these have usually been coexpressed with human RAMPs (hRAMPs). There has been no study of a non-human CL expressed with a RAMP from the same species. It is not known how well these mixed species receptors reflect the pharmacology of the homologous receptors. In turn, this means that there is no reliable information on species variation from exogenously expressed, recombinant receptors.

RAMP2 and RAMP3 are divergent in sequence (Sexton *et al*., 2001), and the regions of RAMP2 and RAMP3 with which AM interacts are not conserved between the two proteins (Kuwasako *et al*., 2001; 2002). This suggests that there could be pharmacological differences between these receptors. Therefore, this study was designed to make a detailed comparison of the highest affinity antagonists available for studying AM/CGRP receptors. At the same time, the effect of species composition on the observed pharmacology was also investigated. AM_{22-52} (Eguchi *et al*., 1994), $CGRP_{8-37}$ (Chiba *et al*., 1989; Dennis *et al*., 1990) and the novel CGRP receptor antagonist BIBN4096BS (Doods *et al*., 2000) were used to inhibit AM responses at AM receptors composed of various combinations of rat or human CL, RAMP2 or RAMP3. A more limited investigation into agonist potency ratios on CL/RAMP3 receptors was also carried out. This work was done using Cos 7 cells which have a null CL/RAMP background, making it an ideal cell line for studying the pharmacology of single populations of AM_1 or AM_2 receptors.

Methods

Cell culture

Cos 7, Rat-2 and L6 cells were cultured in Dulbecco's modified Eagles medium supplemented with 10% foetal bovine serum and 5% penicillin/streptomycin in a humidified 95% air/5% CO_2 atmosphere. The cells were subcultured by removing the growth medium and washing the cells with cell culture-grade phosphate-buffered saline for 1–2 min. The cells were removed from the flasks with a small volume of trypsin/EDTA solution. Fresh growth medium was added to the cell suspension to neutralise the trypsin, and the cells were centrifuged at $500 \times g$ for 5 min. The supernatant was removed, and the cell pellet was resuspended in fresh growth medium. The cells were transferred to fresh flasks, or plated onto 48-well plates.

Cloning of rat RAMP3 (rRAMP3)

rRAMP3 was cloned from a rat lung cDNA library (Invitrogen) using primers based on the published sequence (Oliver *et al*., 2001). These were 5′-CTCGAGATGGCGACCCCGG CACAGCGGCTGCACC-3′ and 5′-GAATTCTCACAGAA GCCGGTCAGTGTGCTTGCTACG-3′. After 30 rounds of polymerase chain reaction (92°C, 60 s; 60°C, 60 s; 72°C, 60 s), using *Pfu* polymerase (Promega), the amplified product was identified as a 0.48 kilobase band on a 1.4% agarose gel. Its identity was confirmed by sequencing (Alta Biosciences, Birmingham, U.K.). The product was subcloned into pcDNA3 using restriction enzyme sites *Eco*RI and *Bam*HI, which were included in the primer design.

Transient transfection

Cells were transfected with various combinations of hRAMP2, hRAMP3, rRAMP3, N-terminally HA epitope-tagged hCL (kindly donated by Dr S.M. Foord, GSK, Stevenage, U.K.) or rCL (Njuki *et al*., 1993), using the calcium phosphate (Clontech) method of transient transfection. Transfections were undertaken essentially according to the manufacturer's instructions, but with minor modifications. Test DNA (1 μg total per well of a 48-well plate) was mixed with sterile water and 2 M calcium chloride solution. This was mixed and left at room temperature for 10 min. The DNA mix was then added dropwise to an equal volume of HEPES-buffered saline. The HEPES-buffered saline was continually agitated as the DNA mix was added to it. This transfection solution was left at room temperature for 30 min. Ten times the volume of the normal growth medium was then added to the transfection solution. The old growth medium was replaced with the transfection solution. After a 5–16 h incubation period, the transfection mix was removed from the cells and replaced with fresh growth

medium. The plates were used for cyclic AMP assay 48–72 h after the medium was replaced.

Assay of cyclic AMP production

The growth medium was removed from the cells and replaced with serum and antibiotic-free Dulbecco's modified Eagle medium containing 500 μM isobutyl methyl xanthine for 30 min. All drugs were diluted in the same medium. Antagonists were added for 15 min before the addition of agonists in the range 1 pM–1 μM for a further 15 min. Cyclic AMP was extracted with ice-cold 95–100% ethanol. Cyclic AMP was measured by radio-receptor assay as previously described (Poyner *et al*., 1992).

Analysis of data

For cyclic AMP studies, the data from each concentration–response curve were fitted to a sigmoidal concentration–response curve to obtain the maximum response, Hill coefficient and EC_{50}, using the fitting routine PRISM Graphpad. From the individual curves, dose ratios were calculated. Where three antagonist concentrations were used, a Schild plot was constructed; after confirming that the slope was not significantly different from unity, it was constrained to 1 to obtain the pK_b. Where only one or two antagonist concentrations were used, an apparent pA_2 was calculated from the formula log[antagonist]−log(dose ratio−1), after first confirming that there were no significant differences in the Hill coefficient or maximum response between the concentration–response curves in the presence and absence of antagonist.

Statistical analysis was carried out by Student's *t*-test, or by one-way ANOVA followed by Tukey's test (where every value was compared against each other), or Dunnett's test (where several values were being compared against a single control). The significance was accepted at $P<0.05$; two-tailed tests were used throughout. All values are quoted as means ± s.e.m.

Materials

Rat AM and human AM_{22-52} were obtained from Bachem (St Helens, Merseyside, U.K.). Human αCGRP (hαCGRP) and human $\alpha CGRP_{8-37}$ ($h\alpha CGRP_{8-37}$) were from Calbiochem (Beeston, Nottingham, U.K.) or Neosystems (Strasbourg, France). [cys(ACM)^{2,7}]αCGRP, rat amylin and rat calcitonin were from Bachem (St Helens, U.K.). [cys(Et)^{2,7}]αCGRP was from Phoenix Pharmaceuticals (Mountain View, CA, U.S.A.), and humanβCGRP (hβCGRP) was from Sigma (Gillingham, Dorset, U.K.). Salmon calcitonin was purchased from Cambridge Research Biochemicals (Northwich, Cheshire, U.K.). All peptides were dissolved in distilled water and stored as aliquots at −20°C or −70°C (AM and AM_{22-52}) in nonstick microcentrifuge tubes (Thermo Life Sciences, Basingstoke, U.K.). BIBN4096BS was a gift from Dr M. Schindler (Boehringer-Ingelheim, Biberach, Germany), and was prepared as previously described (Hay *et al*., 2002). Unless otherwise specified, chemicals were from Sigma or Fisher (Loughborough, U.K.). Cell culture reagents were from Gibco BRL (Paisley, Renfrewshire, U.K.) or Sigma.

Results

Characterisation of baseline receptor expression in Cos 7 cells

Careful characterisation of AM_1 and AM_2 receptors required the use of a batch of cells for transfection studies, which do not express CL or RAMPs endogenously. Cos 7 cells have previously been reported to contain only low levels of RAMPs (Tilakaratne *et al*., 2000). In agreement with this, Figure 1 shows that in cells transfected with CL alone, 100 nM AM (Figure 1a) or concentrations of CGRP up to 1 μM (Figure 1b) failed to cause any increase in cyclic AMP production. This demonstrates that the cells lack any endogenous RAMPs. The cells also failed to respond to these concentrations of AM and CGRP when transfected with RAMP1 or RAMP3 alone, showing the absence of any endogenous CL (Figure 1). By way of positive controls, the cells did respond to AM when transfected with hRAMP3 and rCL, and to CGRP when transfected with hCL and hRAMP1 (Figure 1). These cells were cultured for over 50 passages and tested in this way every few passages. On no occasion were endogenous receptor components evident, making this a suitable cell line for characterising transfected AM_1 and AM_2 receptors.

Attempts were made to further characterise the putative AM receptor L1 (Kapas *et al*., 1995) in transfection experiments using COS 7 cells. However, on no occasion was elevation of

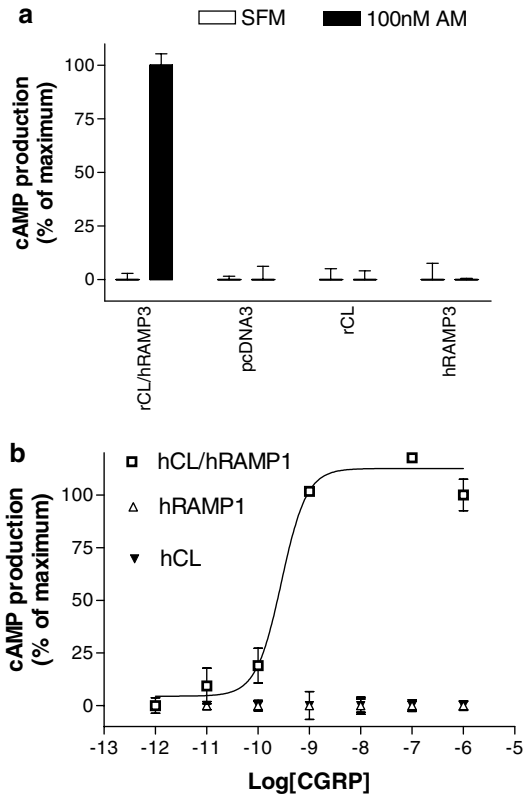

Figure 1 Characterisation of Cos 7 cells, (a) Responses of cells transfected with rCL/hRAMP3, cloning vector (pcDNA3), rCL and hRAMP3, and subsequently challenged with either 100 nM AM or serum-free medium (SFM). (b) Concentration–response curves to hαCGRP in cells transfected with hCL/hRAMP1, hCL and hRAMP1. Points are the mean ± s.e.m. of triplicate determinations. These are representative data from experiments repeated 10 times.

cyclic AMP evident in response to 1 μM of either AM or CGRP (data not shown). The effects of transfection of L1 in the presence of RAMPs were not examined in this study. Concurrent transfection of RAMPs with L1 was reported to be without effect in a previous study (Chakravarty *et al*., 2000).

Characterisation of rRAMP3

rRAMP3 showed three differences from the previously reported sequence (Oliver *et al*., 2001). Codon 128, previously reported as CTG, was TTG; codon 134, previously reported as GGC, was GGG, and codon 137, previously reported as GTG, was GTA. None of these alter the amino acids (i.e. L128, G134 and V137). As *Pfu* polymerase, used for the polymerase chain reaction, has stringent proofreading ability, the probability of obtaining three errors as a result of this process, none of which alter the coding sequence, is very remote. Accordingly, these are likely to be polymorphisms.

Effect of antagonists on AM responses in hCL/hRAMP2-transfected Cos 7 cells

The effects of AM on cyclic AMP responses in hCL/hRAMP2 cotransfected cells in the presence or absence of AM_{22-52}, $CGRP_{8-37}$ and BIBN4096BS are shown in Table 1. In the presence of AM_{22-52}, the concentration–effect curve to AM was shifted to the right in a parallel fashion (Figure 2a). These data were used to generate a Schild plot (Figure 2e). As the slope of the line was not significantly different from unity, the slope was constrained to 1, and a pK_B of 7.34 ± 0.14 ($n = 11$) estimated. $CGRP_{8-37}$ also produced a significant change in the pEC_{50} to AM, with no significant change in Hill coefficient or maximum response (Table 1, Figure 2b). This antagonist was slightly less potent than AM_{22-52} (apparent pA_2 7.04 ± 0.15, $n = 9$, Figure 2b), although the difference was not significant. BIBN4096BS at 10 μM had no significant effect on the response to AM (Table 1).

Effect of antagonists on AM responses in rCL/hRAMP2 Cos 7 cells

The effects of AM on cyclic AMP responses in rCL/hRAMP2 cotransfected cells are shown in Table 1. pEC_{50} values in the presence of AM_{22-52}, $CGRP_{8-37}$ and BIBN4096BS are also shown. In the presence of AM_{22-52}, the concentration–effect curve to AM was shifted to the right in a parallel fashion (Hill slopes; control 0.77 ± 0.5, 1 μM AM_{22-52} 0.5 ± 0.1, 10 μM AM_{22-52} 1.25 ± 0.5). An apparent pA_2 of 6.25 ± 0.17 ($n = 3$) (Figure 2c) was estimated from the shift. There was no significant difference in the pEC_{50} value to AM obtained in the presence of BIBN4096BS or $CGRP_{8-37}$ (Table 1, Figure 2d).

Effect of antagonists on rat AM_1 receptors endogenously expressed in Rat-2 and L6 cell lines

Rat-2 and L6 cell lines have previously been demonstrated to express CL and RAMP2 (Choksi *et al*., 2002), and are therefore good models of rat AM_1 receptors. In L6 cells, AM_{22-52} (1 μM) produced a significant rightward shift in the concentration–effect curve to AM (Table 1, Figure 3a). From this shift, an apparent pA_2 of 7.00 ± 0.05 ($n = 3$) was generated. $CGRP_{8-37}$ (1 μM) was also effective at inhibiting the effects of

Table 1 Actions of antagonists on CL/RAMP2 and CL/RAMP3 complexes

Receptor		$pEC_{50} \pm s.e.m$ (n)									
		AM alone	*+ AM_{22-52} 1 μM*	*+ AM_{22-52} 3 μM*	*+ AM_{22-52} 10 μM*	*AM alone*	*+ $CGRP_{8-37}$ 1 μM*	*+ $CGRP_{8-37}$ 10 μM*	*+ BIBN4096BS AM alone*	*+ BIBN4096BS 10 μM*
hCL/hRAMP2		8.74 ± 0.08 (6)	7.28 ± 0.33 (3)**	6.83 ± 0.14 (4)***	6.43 ± 0.28 (4)***	9.06 ± 0.2 (7)	7.56 ± 0.1 (3)**	7.21 ± 0.2 (6)***	8.61 ± 0.72 (3)	7.64 ± 0.72 (3)
rCL/hRAMP2		8.16 ± 0.14 (6)	8.32 ± 0.42 (3)	–	6.7 ± 0.07 (3)**	8.23 ± 0.3 (4)	–	7.36 ± 0.22 (4)	8.88 ± 0.21 (3)	8.83 ± 0.13 (3)
rCL/rRAMP2	Rat-2	8.76 ± 0.12 (3)	7.45 ± 0.15 (3)*	–	–	8.75 ± 0.11 (3)	7.77 ± 0.01 (3)*	–	8.75 ± 0.11 (3)	9.01 ± 0.09 (3)
	L6	8.70 ± 0.14 (3)	7.64 ± 0.14 (3)*	–	–	8.39 ± 0.08 (4)	7.34 ± 0.01 (3)*	–	8.70 ± 0.14 (3)	8.55 ± 0.13 (3)
hCL/hRAMP3		9.26 ± 0.21 (7)	8.41 ± 0.13 (3)*	8.24 ± 0.15 (3)*	7.34 ± 0.16 (4)***	9.43 ± 0.22 (6)	8.17 ± 0.17 (3)**	7.52 ± 0.19 (6)***	8.49 ± 0.21 (3)	7.72 ± 0.28 (3)
rCL/hRAMP3		8.89 ± 0.25 (6)	8.79 ± 0.47 (3)	–	8.27 ± 0.36 (3)	8.56 ± 0.25 (3)	–	7.06 ± 0.14 (3)**	8.38 ± 0.21 (4)	7.9 ± 0.20 (4)
rCL/rRAMP3		8.30 ± 0.17 (3)	8.06 ± 0.13 (3)	–	7.91 ± 0.24 (3)	8.26 ± 0.72 (5)	7.43 ± 0.07 (5)	7.05 ± 0.39 (5)*	ND	ND

Numbers in parentheses are n values. *, **, *** Significantly different from control pEC_{50}, Dunnett's test at $P < 0.05$, 0.01 and 0.001 levels, respectively. ND, not determined.

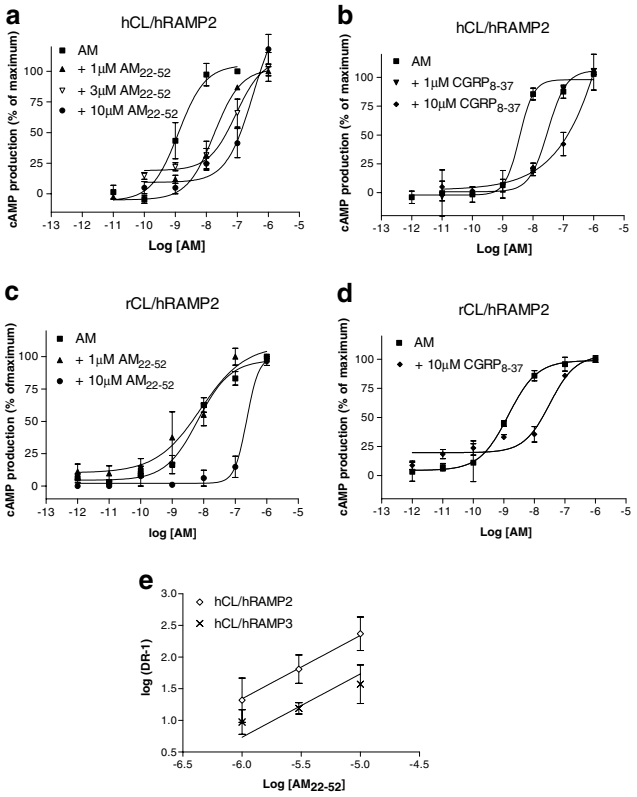

Figure 2 Characterisation of the stimulation of cyclic AMP production by rat AM in Cos 7 cells transfected with CL/RAMP2 combinations. Points are the mean ± s.e.m. of triplicate determinations. Concentration–response curves are representative of three to seven experiments. Data are expressed as the percentage of maximum cyclic AMP production, estimated by fitting each line to a logistic Hill equation, as described in Methods. Maximum cyclic AMP values were 250 ± 20 pmol per 10^6 cells for hCL/hRAMP2, and 450 ± 60 pmol per 10^6 cells for hCL/hRAMP3; basal values were all below 10 pmol per 10^6 cells, (a) hCL/hRAMP2, AM_{22-52}; (b) hCL/hRAMP2, $CGRP_{8-37}$; (c) rCL/hRAMP2, AM_{22-52}; (d) rCL/hRAMP2, $CGRP_{8-37}$; (e) Schild plot, antagonism of AM by AM_{22-52} on hCL/hRAMP2 and hCL/hRAMP3 receptors.

AM, eliciting a significant change in the pEC_{50} ($P<0.05$, Table 1, Figure 3b). An apparent p*A2* value of 7.03 ± 0.12 ($n=3$) was calculated from these data. The effect of CGRP on cyclic AMP in these cells could not be inhibited by 1 μM AM_{22-52} (Figure 3c). BIBN4096BS was unable to antagonise the effects of AM in L6 cells up to concentrations of 10 μM (Table 1, Figure 3a). Slow kinetics of BIBN4096BS have previously been reported (Schindler & Doods, 2002); hence, the incubation time for pretreatment with BIBN4096BS was increased from 15 to 60 min. However, this antagonist was still unable to inhibit the effects of AM. The pEC_{50} values were 8.56 ± 0.32 without BIBN4096BS, compared to 8.30 ± 0.25 (both $n=2$) in the presence of the antagonist. Therefore, the lack of effect of this antagonist in the studies described above is unlikely to be due to the short (15 min) antagonist incubation time. BIBN4096BS inhibited the binding of ^{125}I-iodohistidyl-CGRP to membranes made from COS 7 cells cotransfected with hCL and hRAMP1, with a pK_i of 10.85 ± 0.21. This is in line with its pK_i, on SK-N-MC cells which also express hCL and hRAMP1 (Schindler & Doods, 2002), confirming that the antagonist was active.

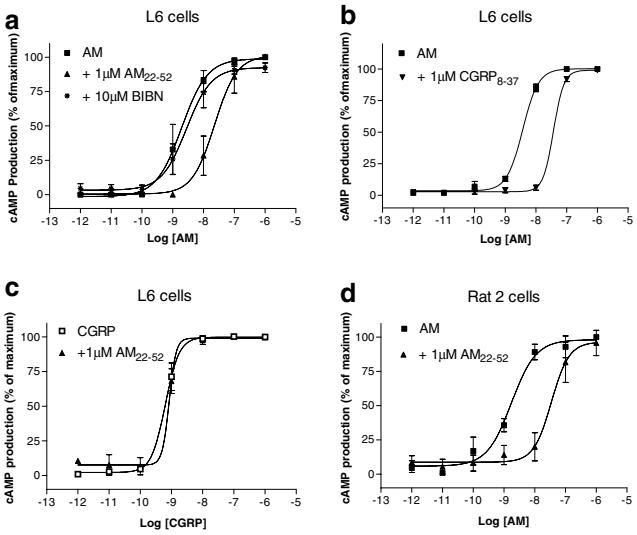

Figure 3 Characterisation of the stimulation of cyclic AMP production by rat AM in L6 and Rat 2 cells (endogenous rCL/rRAMP2). Points are the mean ± s.e.m. of triplicate determinations. Concentration–response curves are representative of three or four experiments. Data are expressed as the percentage of maximum cyclic AMP production, estimated by fitting each line to a logistic Hill equation, as described in Methods. Maximum cyclic AMP values were 290 ± 20 pmol per 10^6 cells for Rat 2 cells, and 2000 ± 300 pmol per 10^6 cells for L6 cells; basal values were all below 10 pmol per 10^6 cells, (a) L6 cells, AM_{22-52} and BIBN4096BS against rAM; (b) L6 cells $CGRP_{8-37}$ against rAM; (c) L6 cells, AM_{22-52} against αCGRP, (d) Rat 2 cells, AM_{22-52} against rAM.

CGRP was inactive on Rat-2 cells at concentrations of up to 1 μM, in accordance with published data (Coppock *et al*., 1999) ($n=3$, data not shown), but AM caused a concentration-dependent stimulation of cyclic AMP production, as shown in Table 1. AM_{22-52} (1 μM) caused a rightward shift in the concentration–effect curve to AM, with an apparent pA_2 of 7.28 ± 0.06 ($n=3$, Table 1, Figure 3d). We have previously demonstrated that the AM response in these cells can be antagonised by 1 μM $CGRP_{8-37}$, but not by 10 μM BIBN4096BS (Hay *et al*., 2002). These data are included in Table 1 for comparison with the data presented here.

Effect of antagonists on AM responses in hCL/hRAMP3 Cos 7 cells

In hCL/hRAMP3 cotransfected cells, the concentration–effect curve to AM was shifted to the right in the presence of AM_{22-52} or $CGRP_{8-37}$ (Table 1, Figure 4a, b). Figure 2e shows the Schild plot generated from the antagonist shifts with AM_{22-52}, from which a pK_B of 6.73 ± 0.14 ($n=10$) was estimated. It was significantly less potent at this receptor ($P<0.01$) than at the hCL/hRAMP2 complex. An apparent pA_2 of 6.96 ± 0.08 ($n=9$, Figure 4b) was generated for $CGRP_{8-37}$. This was not significantly different from its effects at the hCL/hRAMP2 complex. BIBN4096BS was inactive at up to 10 μM.

Effect of antagonists on AM responses in rCL/hRAMP3 Cos 7 cells

In the presence of $CGRP_{8-37}$, the concentration–effect curve to AM was shifted to the right in a parallel fashion (Hill slopes; control 1.3 ± 0. 16, 1 μM $CGRP_{8-37}$ 1.63 ± 0.38, 10 μM $CGRP_{8-37}$,

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