British Journal of Pharmacology (2002) 136, 784–792
www.nature.com/bjp

Comparison of the expression of calcitonin receptor-like receptor (CRLR) and receptor activity modifying proteins (RAMPs) with CGRP and adrenomedullin binding in cell lines

[1,4]Tejal Choksi, [1,4]Debbie L. Hay, [1]Stephen Legon, [2]David R. Poyner, [3]Stefanie Hagner, [1]Stephen R. Bloom & *,[1]David M. Smith

[1]Department of Metabolic Medicine, Imperial College School of Medicine, Hammersmith Hospital, Du Cane Road, London W12 0NN; [2]School of Life Sciences, Aston University, Aston Triangle, Birmingham B4 7ET and [3]Institute of Physiology, Philipps-University, Deutschhausstrasse 2, D35037, Marburg, Germany

1 The calcitonin receptor-like receptor (CRLR) and specific receptor activity modifying proteins (RAMPs) together form receptors for calcitonin gene-related peptide (CGRP) and/or adrenomedullin in transfected cells.

2 There is less evidence that innate CGRP and adrenomedullin receptors are formed by CRLR/RAMP combinations. We therefore examined whether CGRP and/or adrenomedullin binding correlated with CRLR and RAMP mRNA expression in human and rat cell lines known to express these receptors. Specific human or rat CRLR antibodies were used to examine the presence of CRLR in these cells.

3 We confirmed CGRP subtype 1 receptor ($CGRP_1$) pharmacology in SK-N-MC neuroblastoma cells. L6 myoblast cells expressed both $CGRP_1$ and adrenomedullin receptors whereas Rat-2 fibroblasts expressed only adrenomedullin receptors. In contrast we could not confirm $CGRP_2$ receptor pharmacology for Col-29 colonic epithelial cells, which, instead were $CGRP_1$-like in this study.

4 L6, SK-N-MC and Col-29 cells expressed mRNA for RAMP1 and RAMP2 but Rat-2 fibroblasts had only RAMP2. No cell line had detectable RAMP3 mRNA.

5 SK-N-MC, Col-29 and Rat-2 fibroblast cells expressed CRLR mRNA. By contrast, CRLR mRNA was undetectable by Northern analysis in one source of L6 cells. Conversely, a different source of L6 cells had mRNA for CRLR. All of the cell lines expressed CRLR protein. Thus, circumstances where CRLR mRNA is apparently absent by Northern analysis do not exclude the presence of this receptor.

6 These data strongly support CRLR, together with appropriate RAMPs as binding sites for CGRP and adrenomedullin in cultured cells.

British Journal of Pharmacology (2002) **136,** 784–792

Keywords: CGRP; adrenomedullin; CRLR; RAMP; SK-N-MC neuroblastoma cells; Col-29 cells; L6 myoblasts; Rat-2 fibroblasts

Abbreviations: α-CGRP, calcitonin gene-related peptide; $CGRP_1$ and $CGRP_2$, CGRP receptor subtypes; CRLR, calcitonin receptor-like receptor; [Cys(ACM)^{2,7}]α-CGRP, [acetimidomethyl-Cys^{2,7}]α-CGRP; [Cys(Et)^{2,7}]α-CGRP, [ethylamide-Cys^{2,7}]α-CGRP; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IBMX, isobutylmethylxanthine; RAMP, receptor activity modifying protein

Introduction

Adrenomedullin and calcitonin gene-related peptide (CGRP) are structurally related members of the calcitonin family of regulatory peptides, which share many overlapping biological functions such as extremely potent vasodilatory behaviour (Brain & Cambridge, 1996; Hinson *et al*., 2000). CGRP receptors are of particular interest as anti-migraine targets since CGRP release and the onset of head pain have been clearly correlated (Edvinsson, 2001; Doods, 2001). Studies with adrenomedullin gene knock-out mice demonstrate that this peptide and its receptors are essential for the formation of a functional cardiovascular system (Hay & Smith, 2001; Caron & Smithies, 2001).

High affinity binding sites, specific for either CGRP or adrenomedullin, have been demonstrated in various tissues and cell lines (Henke *et al*., 1987; Sexton *et al*., 1986; Poyner *et al*., 1992; Owji *et al*., 1995; Coppock *et al*., 1999). CGRP receptors have been divided into two subtypes ($CGRP_1$ and $CGRP_2$) based, predominantly, on the ability of the antagonist $CGRP_{8-37}$ to inhibit the effects of CGRP in functional studies; binding studies are not able to distinguish these putative subtypes (Dennis *et al*., 1989; 1990; Rorabaugh *et al*., 2001). At $CGRP_1$ receptors this CGRP fragment is a potent antagonist whereas $CGRP_2$ receptors are more resistant to its effects (Dennis *et al*., 1989; 1990; Chiba *et al*., 1989). Two non-cyclic analogues of CGRP, α-[acetimi-

*Author for correspondence at: AstraZeneca, Mereside, Alderley Park, Macclesfield, Cheshire, SK10 4TG;
E-mail: dave.smith@astrazeneca.com
[4]The first two authors contributed equally to this study

domethyl-Cys^{2,7}]hCGRP ([Cys(ACM)^{2,7}]α-CGRP) and α-[ethylamide-Cys^{2,7}]hCGRP ([Cys(Et)^{2,7}]α-CGRP) have been proposed as $CGRP_2$-specific agonists (Dennis *et al*., 1989; Dumont *et al*., 1997), however their specificity has recently been questioned (Wu *et al*., 2000; Wisskirchen *et al*., 1998). Critical to the development of specific ligands for these receptors and to fully understand the biology of these peptides is a determination of the molecular nature of all CGRP and adrenomedullin binding sites.

Though the calcitonin receptor was cloned in 1991 (Lin *et al*., 1991), CGRP and adrenomedullin receptors have proven difficult to identify. In 1995 an orphan receptor known as L1 was reported to be an adrenomedullin receptor and a related receptor, RDC-1, to be a $CGRP_1$ receptor (Kapas *et al*., 1995; Kapas & Clark, 1995). However this work has been difficult to reproduce (Kennedy *et al*., 1998). Recent evidence suggests that both adrenomedullin and CGRP bind to the same seven transmembrane domain receptor, the calcitonin receptor-like receptor (CRLR, Njuki *et al*., 1993; Fluhmann *et al*., 1995). Interestingly, the specificity of CRLR for these peptides is conferred by a novel family of single transmembrane proteins, termed receptor-activity modifying proteins (RAMPs, McLatchie *et al*., 1998). Concurrent CRLR and RAMP expression is essential for the formation of functional receptors. Furthermore, RAMPs appear to be closely associated with CRLR at the cell surface and may contribute part of the peptide binding site (Hilairet *et al*., 2001). CRLR functions as a $CGRP_1$ receptor when co-expressed with RAMP1 but as an adrenomedullin-preferring receptor in the presence of either RAMP2 or RAMP3 in transfection studies using HEK 293T cells (McLatchie *et al*., 1998). This basic hypothesis has been supported by further transfection studies from a number of groups (Muff *et al*., 1998; Kamitani *et al*., 1999; Chakravarty *et al*., 2000), however more information is required concerning the relevance of these receptor combinations to the physiological actions of CGRP and adrenomedullin. Whether $CGRP_2$ receptors are also formed by CRLR and RAMP combinations is not yet known. IUPHAR guidelines are that the complexes formed by CRLR/RAMP2 and CRLR/RAMP3 should be known as AM_1 and AM_2 receptors; however these entities show similar pharmacological properties with respect to their affinities for $CGRP_{8-37}$ (Poyner *et al*., 2002).

We recently hypothesized that CRLR/RAMP combinations account for the majority of observed CGRP and adrenomedullin binding in rat tissues. We found a significant correlation between CGRP binding and RAMP1 mRNA expression but the correlation between adrenomedullin binding and RAMP2 expression was not significant (Chakravarty *et al*., 2000). As an extension to this study we have evaluated the relationship between CGRP and adrenomedullin binding and CRLR/RAMP transcript levels in immortalized cell lines. These cell lines have previously been reported to express various combinations of CGRP and/or adrenomedullin receptors and should represent a less complex system than tissues for correlating receptor type with CRLR and RAMP mRNA (Longmore *et al*., 1994; Coppock *et al*., 1996; 1999; Kirkland, 1985). We have also established the presence of CRLR protein in each of these cell lines using specific CRLR antibodies (Hagner *et al*., 2001).

Methods

Materials

Rat adrenomedullin and rat [Tyr^0]α-CGRP were obtained from Peninsula Laboratories Inc (St Helens, Merseyside, U.K.). Rat α-CGRP and human α-$CGRP_{8-37}$ were custom synthesized by ASG University (Szedgel, Hungary) and Dr P.G.H. Byfield (Haemostasis Research Unit, Clinical Sciences Centre, Hammersmith Hospital, London, U.K.) respectively. [Cys(ACM^{2,7}]α-CGRP was from Peninsula and [Cys(Et)^{2,7}]α-CGRP was from Phoenix Pharmaceuticals Inc (Mountain View, CA, U.S.A.). All peptides were checked for correct molecular weight by mass spectroscopy. Na[^{125}I], polyvinylidene difluoride (PVDF) membrane, ECL Plus reagent and Hyperfilm ECL were supplied by Amersham Pharmacia Biotech (Little Chalfont, Bucks, U.K.). Iodogen reagent was supplied by Pierce (Rockford, Illinois, U.S.A.). Unless otherwise specified chemicals were from Sigma Chemical Co (Sigma-Aldrich, Gillingham, Dorset, U.K.) and cell culture materials from Gibco BRL (Life Technologies, Paisley, Renfrewshire, U.K.).

Peptide iodination

Rat adrenomedullin and rat [Tyr^0]α-CGRP were iodinated by the Iodogen method as previously described (Owji *et al*., 1995; Bhogal *et al*., 1993). Adrenomedullin (12.5 μg, 2 nmoles) in 10 μl of phosphate buffer 0.2 M, pH 7.2 was incubated with 37 MBq of ^{125}NaI and 10 μg of Iodogen reagent for 4 min at 22°C. The ^{125}I-labelled peptide was purified by reverse phase HPLC (Waters C_{18}Novopak, Millipore, Milford MA, U.S.A.) using a 15–40% acetonitrile/water/0.05% trifluoroacetic acid gradient. The specific activity of the label was estimated as 10 Bq fmol^{-1} by comparison of simultaneous saturation and competition binding data. Rat [Tyr^0]α-CGRP was iodinated by a similar procedure except 12 μg (2.4 nmoles) were incubated on ice for 5 min together with the ^{125}NaI and the products separated on a 20–50% acetonitrile/water/0.05% trifluoroacetic acid gradient. The specific activity of the label was estimated as 36 Bq fmol^{-1} by radio-immunoassay (Upton *et al*., 1997). Fractions showing binding were aliquoted, freeze-dried and stored at −80°C.

Cell culture

SK-N-MC human neuroblastoma (a gift from Prof S. Nahorski, University of Leicester, widely considered to express the 'standard' $CGRP_1$ receptor (Longmore *et al*., 1994)), Rat-2 fibroblast (European Collection of Animal Cell Cultures (ECACC), previously reported to only express adrenomedullin receptors (Coppock *et al*., 1999)), L6G8C5 rat myoblasts (L6-1 cells from Aston University stock and L6-2 from ECACC, shown to express both CGRP and adrenomedullin receptors (Coppock *et al*., 1996)) and Col-29 human colon epithelium cells (previously reported to express $CGRP_2$ receptors, kindly provided by Dr S.C. Kirkland, Department of Histopathology, ICSM, Hammersmith Hospital, London, U.K. (Kirkland, 1985; Cox & Tough, 1994; Poyner *et al*., 1998)) were cultured as previously described (Morgan *et al*., 1998; Coppock *et al*., 1996; 1999; Poyner *et al*., 1998). The cells were grown to confluence and were used

for whole cell binding, cAMP assay, RNA and protein extraction.

Whole cell adrenomedullin and CGRP ligand binding assays

Confluent cell monolayers (approximately 2×10^5 cells per well), grown on poly-D-lysine coated 24-well plates, were washed once with binding buffer (mM): HEPES 20, pH 7.4, $MgCl_2$ 5, NaCl 10, KCl 5, EDTA 1; phosphoramidon 1 μM and 0.1% w v^{-1} BSA, and incubated for 60 min at 4°C for adrenomedullin or 22°C for CGRP in binding buffer containing 1000 Bq of ^{125}I-adrenomedullin or ^{125}I-[Tyr^0]α-CGRP. Unbound label was removed by aspiration of binding buffer and cells were washed three times with ice-cold binding buffer. Cells were solubilized in 500 μl of NaOH 0.1 M and counted in a gamma counter. Non-specific binding was determined in the presence of excess unlabelled rat adrenomedullin (100 nM) or α-CGRP (1 μM). These concentrations are sufficient to occupy >99% of the receptors. All binding was performed under equilibrium conditions (Bhogal *et al*., 1993; Owji *et al*., 1995).

Assay of cAMP production

Cells were grown to confluence and prior to experimentation were incubated with serum free medium for 2 h 30 min to minimize unstimulated cAMP levels. Cells were then incubated in medium containing isobutylmethylxanthine (IBMX) 0.5 mM for 30 min and all media thereafter also contained IBMX. For agonist studies, cells were incubated with the required agonist for 15 min and cAMP extracted in ethanol for 1 h at 4°C. Cyclic AMP was then measured by radio-immunoassay using Amersham SPA kits (Amersham Pharmacia Biotech) or NEN flashplate kits (NEN life Science Products, Hounslow, Middlesex, U.K.) (Abbott *et al*., 2000). In experiments where the antagonist $CGRP_{8-37}$ was used, following serum starvation (as above) cells were incubated with antagonist for 5 min prior to addition of agonist (plus antagonist) for 15 min. Cyclic AMP was then extracted and measured as above. Concentration effect curves were analysed using Prism 3 (GraphPad Software, Inc., San Diego, CA, U.S.A.). From these, dose-ratios were calculated (using the curves obtained in the presence of 30, 100 and 300 nM antagonist). These were used to produce a Schild plot; the slopes were calculated by linear regression and found not to be different from unity. They were then constrained to 1 to calculate pK_b values.

Northern blot analysis

Total RNA was prepared and analysed on formaldehyde agarose gels as previously described (Sharma *et al*., 1992). The rat probes have also been described in a previous publication (Chakravarty *et al*., 2000). For human probes, full length myc-tagged CRLR (1467 bp), and full length RAMP1 (501 bp), RAMP2 (588 bp) and RAMP3 (508 bp) cDNAs were obtained as a gift from Dr S.M. Foord, (Glaxo Wellcome Research and Development, Stevenage, Herts, U.K. (McLatchie *et al*., 1998)). Northern analysis was performed as described previously (Chakravarty *et al*., 2000). Briefly, the insert of each clone was labelled using random primer synthesis (^{32}P incorporation efficiency 1.5×10^{-9} μg^{-1}) and equivalent amounts of radiolabelled probe were used for hybridizations. Northern analysis was quantified using a PhosphorImager with ImageQuant software (Molecular Dynamics Inc, Sunnyvale, CA, U.S.A.). The blots were then probed successively for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin mRNA and finally for the total amount of polyA$^+$ RNA in each track using a 45^{mer} oligo-dT probe labelled by tailing with a single ^{32}P-dCTP residue. For each standard, hybridization in each tissue was expressed as a percentage of the total and the mean figures for the three standards were used to normalize the values for the test probes.

Anti-CRLR antibodies and Western blot analysis

Two affinity-purified rabbit polyclonal antibodies were employed in these investigations. One (MR567) was raised to the carboxy-terminus of human CRLR and has been characterized elsewhere (Hagner *et al*., 2001). The other antibody (MR003) was raised in an identical manner to MR567 but to the carboxy-terminus of rat CRLR. This antibody has been shown to detect the rat sequence following its transfection into HEK293 cells by both Western blot and immunohistochemical analysis (D.L. Hay *et al*., unpublished observations). MR567 was used at a dilution of 1 : 200 and MR003 at a dilution of 1 : 500. Total protein was prepared in the following manner; cells were harvested and centrifuged at $500 \times g$ for 5 min. The resulting pellets were incubated in cell lysis buffer (1% v v^{-1} Triton X-100, 10% v v^{-1} glycerol, (mM): NaCl 150, NaF 50, HEPES 20, pH 7.5) containing protease inhibitors, on ice for 30 min. The resulting cell lysate was centrifuged at 10,000 r.p.m. in a bench-top centrifuge for 20 min at 4°C. The supernatant was collected and the protein concentration determined. The supernatants were subjected to 10% SDS–PAGE, then transferred to PVDF membranes using a wet transfer method (25 V, overnight, 4°C). Non-specific binding was blocked by incubating the PVDF membrane in phosphate-buffered saline (PBS) containing 5% non-fat milk for 1 h. Membranes were then incubated at room temperature for 1 h with primary antibody in PBS containing 0.25% non-fat milk and 0.02% Tween 20 and then with a horseradish peroxidase-linked secondary antibody (in PBS, 5% milk, 0.02% Tween 20) for 1 h at room temperature. Following each incubation with antibody, the membrane was washed four times (at 15 min intervals) with PBS containing 0.02% Tween 20. Bound antibodies were detected using the ECL Plus Western blotting detection kit and autoradiography was performed with Hyperfilm ECL.

Statistical analysis

Statistical analysis was undertaken using the Students *t*-test (two-tailed).

Results

Analysis of receptor type in SK-N-MC, Rat-2, L6 and Col-29 cells

Since we have found that receptor expression in cell lines can vary, it was essential to verify the receptor types present in

the exact cells used in this study thus allowing accurate comparisons of pharmacology, mRNA and protein expression to be made.

Analysis of receptor type by whole cell binding assay Whole cell binding for ^{125}I-[Tyr^0]α-CGRP and ^{125}I-adrenomedullin was performed for all cell lines (Table 1). For most of the cell lines, CGRP and adrenomedullin binding was as previously reported. SK-N-MC cells had clear CGRP binding but no adrenomedullin binding. Rat-2 cells bound adrenomedullin but not CGRP. Col-29 cells showed CGRP binding but no adrenomedullin binding. The amount of adrenomedullin binding in L6-1 cells was similar to what we have previously reported but CGRP binding in this study was less than previously shown (Coppock *et al*., 1996). Similar results were obtained with L6-2 cells. There was a considerable difference in the non-specific binding of the radioligands between these two populations of L6 cells; the reason for this is unknown.

Analysis of receptor type by cAMP assay The EC_{50} values for stimulation of cAMP by CGRP in SK-N-MC and Col-29 cells were 1.5 and 2.1 nM respectively (Figure 1a,b). Maximal cAMP stimulation achieved by CGRP in these cell lines was 42.8 ± 1.0 and 8.6 ± 0.3 pmol per well for SK-N-MC and Col-29 cells respectively. This approximately 5 fold difference between the cell lines correlates with the density of CGRP receptors (Table 1). Analysis of inhibition of CGRP stimulated cAMP production by $CGRP_{8-37}$ showed similar characteristics for SK-N-MC and Col-29 cells (Figure 1a,b). When these data were used to calculate a Schild plot (Figure 1c), the slopes of the lines were not significantly different from unity (SK-N-MC, 1.01 ± 0.06; Col 29, 1.27 ± 0.03). When the lines were constrained to 1, the resulting pK_b values were not significantly different (SK-N-MC, 8.14 ± 0.02; Col 29, 8.19 ± 0.08). Since we would expect the pK_b value for $CGRP_{8-37}$ in Col-29 cells to be at least one log unit lower than in SK-N-MC cells (Poyner *et al*., 1998), in this circumstance the CGRP receptor in these Col-29 cells could not be confirmed as $CGRP_2$. This result is contrary to published data (Cox & Tough, 1994) and indeed our own data (Poyner *et al*., 1998) so we further investigated these cells using the putative $CGRP_2$ selective agonists [Cys(ACM)^{2,7}]α-CGRP and [Cys(Et)^{2,7}]α-CGRP. [Cys(ACM)^{2,7}]α-CGRP weakly stimulated cAMP in both cell lines. In SK-N-MC cells, 100 nM [Cys(ACM)^{2,7}]α-CGRP produced weak but significant cAMP stimulation from a basal level of 1.41 ± 0.18 to 5.91 ± 0.96 pmol per well ($P > 0.05$, $n = 3$) in agonist treated wells (not shown). In Col-29 cells, 100 nM [Cys(ACM)^{2,7}]α-CGRP produced a non-significant increase in cAMP from a basal level of 2.47 ± 0.35 to 2.94 ± 0.55 pmol per well ($n = 3$) in agonist treated wells (not shown). [Cys(Et)^{2,7}]α-CGRP was a more potent agonist in both cell types but there was only a 2 fold difference between the two cell lines with an EC_{50} of 67 nM in SK-N-MC cells and 152 nM in Col-29 cells (Figure 1d). The similarity of responses with these agonists also suggests that the Col-29 cells used in this study have a $CGRP_1$ rather than $CGRP_2$ phenotype.

Detection of CRLR and RAMP mRNA

Total RNA was extracted from all cells in parallel with the receptor characterization for that cell type to ensure that the binding or cAMP results observed could be correlated with the mRNA data. The RNA was analysed on four parallel Northern blots and probed with CRLR, RAMP1, RAMP2 and RAMP3 cDNA probes. The signals were normalized by probing successfully with GAPDH, β-actin and oligo dT_{45} as controls for RNA loading. The figures for the target RNAs have been normalized using the mean of these three controls (Table 2). Rat lung total RNA was used as a positive control on all blots as this tissue expresses CRLR, RAMP1 and RAMP2 and both adrenomedullin and CGRP binding (Chakravarty *et al*., 2000).

SK-N-MC cells at passage 5 showed the highest levels of CRLR and RAMP1 mRNA and low levels of RAMP2 (Figure 2 and Table 2). Note the characteristic multiple bands seen in human CRLR mRNA compared with the single band seen in rat CRLR mRNA (Chakravarty *et al*., 2000) (Figures 2 and 3). This may be due to either alternative promoters or splicing of the mRNA but this needs further investigation. Col-29 cells showed expression of CRLR and RAMP1 mRNA but only low levels of RAMP2 mRNA (Figure 2). Rat-2 cells showed some CRLR mRNA expression, no RAMP1 mRNA but an abundance of RAMP2 mRNA (Figure 3, Table 2). In contrast to these three cell lines we could not detect CRLR in L6-1 cells. However RAMP1 and RAMP2 mRNA were present (Figure 3, Table 2). We chose to investigate a second source of L6 cells for CRLR expression to evaluate whether this apparent lack of CRLR was specific to this batch of cells. These cells, designated L6-2, showed a different pattern of expression to L6-1 cells with CRLR, RAMP1 and 2 all present at moderate levels (Figure 3, Table 2). We found no expression of RAMP3 mRNA in any of the cell lines we examined in this study (results not shown).

Detection of CRLR protein

In order to confirm the presence or absence of CRLR in our cell lines we used specific human or rat anti-CRLR antibodies

Table 1 ^{125}I-CGRP and ^{125}I-adrenomedullin binding in SK-N-MC, Col-29, L6-1, L6-2 and Rat-2 cell lines

	^{125}I-adrenomedullin		*^{125}I-CGRP*	
	Total binding ± s.e.mean (fmols per 10^6 cells)	*NSB ± s.e.mean* (fmols per 10^6 cells)	*Total binding ± s.e.mean* (fmols per 10^6 cells)	*NSB ± s.e.mean* (fmols per 10^6 cells)
SK-N-MC	2.3 ± 0.3	2.7 ± 0.2	5.2 ± 0.3	1.3 ± 0.1
Col-29	2.6 ± 0.2	2.2 ± 0.2	1.5 ± 0.1	0.7 ± 0.03
L6-1	29.7 ± 0.8	5.6 ± 0.3	2.4 ± 0.1	0.9 ± 0.03
L6-2	35.0 ± 2.0	21.0 ± 1.0	3.5 ± 0.3	2.6 ± 0.3
Rat-2	10.8 ± 0.5	3.6 ± 1.2	0.13 ± 0.03	0.13 ± 0.01

NSB, non-specific binding. Results are expressed as s.e.mean of quadruplicate determinations.

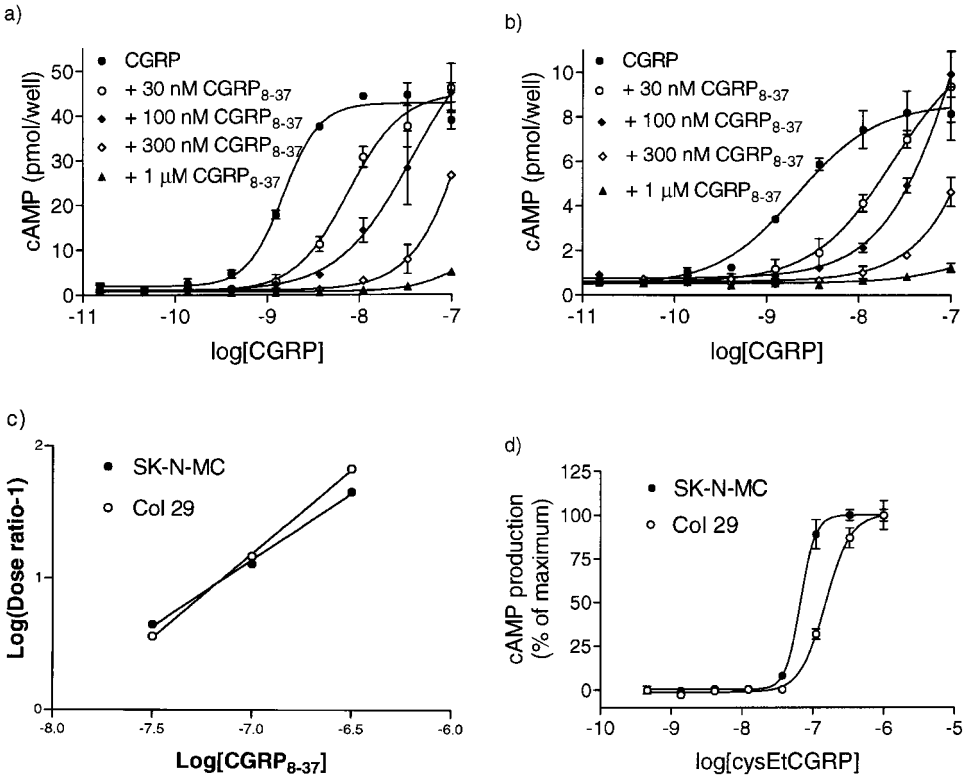

Figure 1 Stimulation of cAMP production in SK-N-MC (a) and Col-29 (b) cells by CGRP in the absence or presence of different $CGRP_{8-37}$ concentrations. Cyclic AMP production is shown as pmol cAMP per well. Points represent the mean and s.e.mean of three determinations. (c) Schild plot showing the effects of $CGRP_{8-37}$ on SK-N-MC and Col-29 cells. This illustrates the case where the slope of the plot has not been constrained to unity. (d) Effect of $[Cys(Et)^{2,7}]\alpha$-CGRP on cAMP production in SK-N-MC and Col-29 cells. Cyclic AMP production is shown as a per cent of the maximal stimulation level in those cells. Points represent the mean and s.e.mean of three determinations.

Table 2 Northern analysis of SK-N-MC (passage numbers 5 or 25), L6-1, L6-2 or Rat-2 cells

Cell type	*CRLR* (arbitrary units)	*RAMP1* (arbitrary units)	*RAMP2* (arbitrary units)
SK-N-MC (5)	2652	8260	384
SK-N-MC (25)	0	1392	192
L6-1	0	2956	4792
L6-2	963	318	916
Rat-2	250	34	6134
Rat lung	22978	1405	48238

Values are arbitrary PhosphorImager units after normalization. Values of zero are indicative of a normalized mRNA value equal to or less than background levels for the CRLR probe. Total RNA (25 μg) was probed in all cases.

raised against the C-terminal portion of the receptor. In contrast to our mRNA data we found that all of the cell lines, including L6-1 cells were positive for CRLR protein expression (Figure 4a,b). Whereas the human antibody detected only a single band at approximately 80 kD, the rat antibody detected multiple bands. In the initial characterization of the human CRLR antibody on SK-N-MC cells a single band of 59 kDa was detected (Hagner *et al.*, 2001). Subsequent studies in human tissues have shown that this antibody predominantly detects a band of approximately 70 kDa (S. Hagner, unpublished observations), broadly in line with our determination. It is possible that the discrepancy in molecular weights relates to differences in the preparation of the protein samples or is another example of the inherent variability of the SK-N-MC cells. The rat band at approximately 75 kDa corresponds well to the band found in our SK-N-MC cells. Furthermore, the size of this rat band agrees with published cross-linking data (Coppock *et al.*, 1996; 1999).

Effect of continuous passage on SK-N-MC binding, cAMP signal and receptor mRNA level

During this and other studies using SK-N-MC cells we noticed that binding and cAMP activity in response to agonist stimulation are lost as these cells undergo continuous passage. The difference in CGRP binding and cAMP activity in response to CGRP over a period of 20 passages is shown in Figure 5a,b. At passage 25 SK-N-MC cells have negligible CGRP binding compared to passage 5 cells (Figure 5a; the difference in total binding between the two passages of cells was significant ($P<0.001$) whereas there was no significant difference between non-specific binding). Figure 5b shows dose response analysis of cAMP production in response to CGRP in low and high passages of SK-N-MC cells. The EC_{50} of cAMP production in low passage SK-N-MC cells was 0.2 nM whereas in high passage cells the EC_{50} value was in excess of 1 μM. Both populations of cells showed a significant elevation of cAMP ($P<0.05$) when challenged with 10 μM forskolin. mRNA was prepared from both

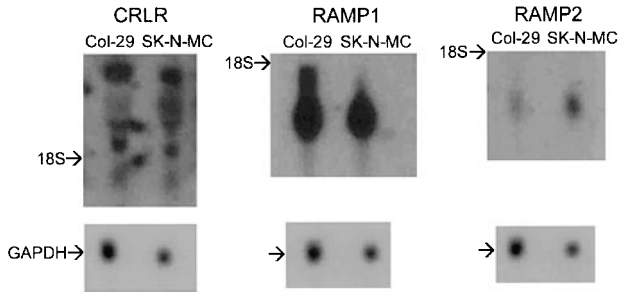

Figure 2 Northern analysis of Col-29 and SK-N-MC cells showing (from left to right) CRLR, RAMP1, and RAMP2 mRNA. Note that human CRLR mRNA appears as multiple bands, as discussed in the text. The blots were also probed with GAPDH and the resulting autoradiogrphs are aligned under the original blots. The position of the 18S rRNA band, which was used as a marker, is shown on each blot.

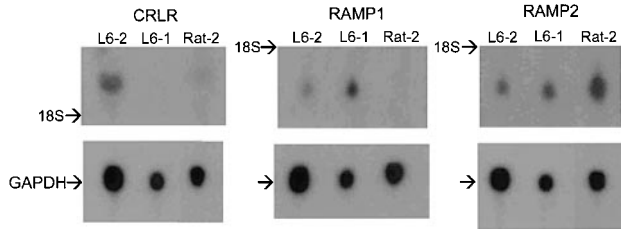

Figure 3 Northern analysis of L6-2, L6-1 and Rat-2 cells showing (from left to right) CRLR, RAMP1 and RAMP2 mRNA. These blots were also probed with GAPDH and the resulting autoradiographs are aligned under the original blots. The position of the 18S rRNA band, which was used as a marker, is shown on each blot.

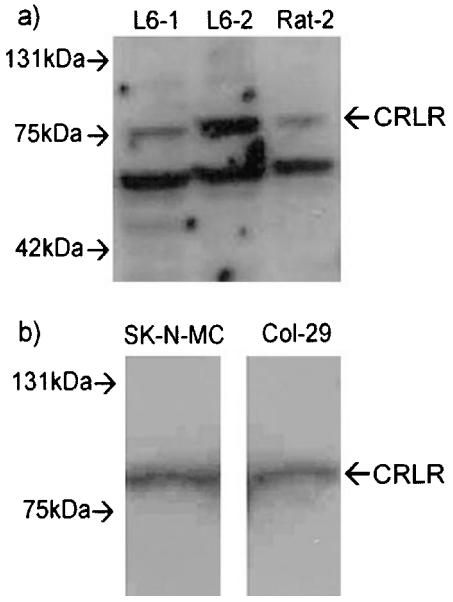

Figure 4 Western blots of rat (a) or human (b) cell line total protein. (a) Twenty μg of total protein analysed on a 10% SDS–PAGE gel was exposed to an anti-rat CRLR antibody. A band of 75 kDa, representing rat CRLR was detected in L6-1, L6-2 and Rat-2 cells. (b) Total protein (20 μg) from human cell lysates was analysed in the same way as for the rat cells. A single band of 80 kDa representing human CRLR was detected in SK-N-MC and Col-29 cells. Molecular weight markers, shown on the left of the blots are: β-galactosidase, 131 kDa; bovine serum albumin, 75 kDa and carbonic anhydrase, 42 kDa.

passages of cells as described above. We found that CRLR mRNA was reduced to below our limits of detection between passages 5 and 25 and that RAMP1 was reduced by 83.1% (Figure 5c and Table 2). RAMP2 mRNA also decreased but to a lesser extent (50%).

Discussion

There is increasing evidence to support the original hypothesis of Foord and co-workers that CRLR with RAMP1 forms a $CGRP_1$ receptor and CRLR with RAMP2 or RAMP3 forms an adrenomedullin preferring receptor in transfected cell lines (McLatchie *et al*., 1998; Muff *et al*., 1998; Kamitani *et al*., 1999; Chakravarty *et al*., 2000). Specific antibodies to CRLR and RAMPs and mRNA studies are now being used to evaluate the role of these receptor components in tissues and cell lines (Hagner *et al*., 2001; Nikitenko *et al*., 2001; Oliver *et al*., 2001; Ueda *et al*., 2001). Recently, we presented evidence showing a strong correlation between CRLR and RAMP1 mRNA expression and CGRP binding in a selection of rat tissues (Chakravarty *et al*., 2000). However, the association of CRLR with RAMP2 or 3 and adrenomedullin binding was less convincing (Chakravarty *et al*., 2000). A potential explanation of these results is that a proportion of adrenomedullin binding is not associated with CRLR/RAMP2 or 3. Receptor heterogeneity within tissues also adds complication. In this study we have used homogenous cell lines to circumvent this issue and to determine whether CRLR and RAMPs are responsible for generating CGRP and/or adrenomedullin binding sites in cell lines commonly used as models for these receptors.

Although it is often considered that cultured cells represent dependable systems for analysis of receptors there can be considerable variability of apparently the same cell line between laboratories. For example, in our hands HEK293 cells express calcitonin receptors (Han *et al*., 1997) but in others this is not the case (Findlay *et al*., 1994). In the RAMP field, the background of a particular cell type can contribute greatly to the final observed receptor phenotype following transfection and this background can vary between batches of cells (Tilakaratne *et al*., 2000). As shown clearly with SK-N-MC cells in this study, receptors can also be lost or gained over time (Sulistiyani & St Clair, 1991; Makino *et al*., 2001). Hence, we assessed the receptor type and extracted RNA and protein from the same cells, on the same day. Our data cannot be used to make general assumptions on the characteristics of a cell line, only on these particular batches of cells at defined passage.

Here we show that, in accordance with the proposed hypothesis, $CGRP_1$ receptor binding sites in SK-N-MC cells are likely to be formed by CRLR and RAMP1. Low levels of RAMP2 in these cells and higher levels of RAMP1 are associated with no adrenomedullin binding but good CGRP binding. This is also supported by RAMP1 being dominant in binding phenotype (Buhlmann *et al*., 1999). Interestingly, McLatchie *et al*. (1998) found equivalent levels of RAMP1 and 2 in SK-N-MC cells, another illustration of the intrinsic variability of cell lines. In our study CRLR mRNA is abundant and CRLR protein is present. To add extra weight

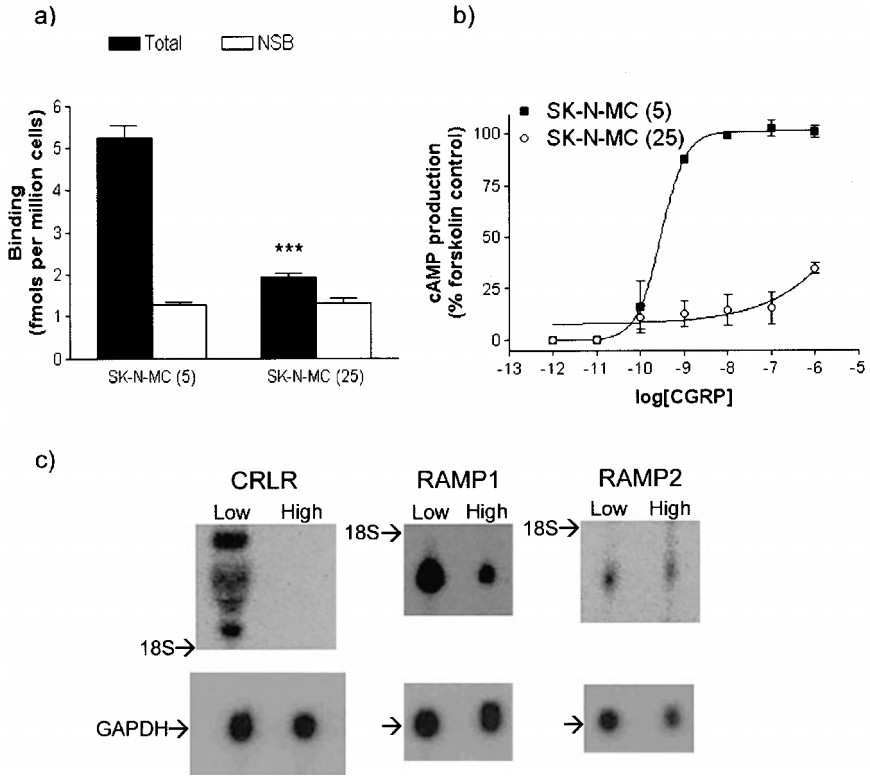

Figure 5 The effect of passage number on ^{125}I-CGRP binding (a), cAMP stimulation (b) and CRLR, RAMP1 and RAMP2 mRNA levels (c) in SK-N-MC cells. (a) Total and non-specific binding (NSB) were measured in the cells as described in Methods. Passage number is shown in parentheses. Each bar represents the mean and s.e.mean of quadruplicate determinations. (b) Cyclic stimulation by CGRP in SK-N-MC cells at passage 5 or 25 expressed as a percentage of the response to 10 μM forskolin (assay points represent the mean of 2 to 3 separate, duplicate experiments, vertical lines are s.e.mean). The cAMP production in response to forskolin was 30 pmol per well (passage 5) and 6.5 pmol per well (passage 25). (c) Northern analysis of low passage (5) or high passage (25) SK-N-MC cells showing (from left to right) CRLR, RAMP1 and RAMP2 mRNA. These blots were also probed with GAPDH and the resulting autoradiographs are aligned under the original blots. The position of the 18S rRNA band, which was used as a marker, is shown on each blot.

to this finding, over 20 passages, CRLR mRNA is reduced to below our detection limit in accordance with a dramatic loss of RAMP1 mRNA and a large reduction of CGRP binding and cAMP production.

The behaviour of the Col-29 cells in this study stands in contrast to the $CGRP_2$-like phenotype that we and others have previously found (Poyner *et al*., 1998; Cox & Tough, 1994). Perhaps significantly there were differences between the behaviour of the cells in the hands of Cox and Tough, who found that 3 μM $CGRP_{8-37}$ had no effect on the response to CGRP and ourselves, who found that this concentration was able to antagonize CGRP. Subsequent to the study presented here we have examined the pharmacology of another batch of these cells. These showed a significant difference in affinity for $CGRP_{8-37}$ compared to SK-N-MC cells (pK_b values; 7.34 ± 0.19, $n=7$ versus 8.35 ± 0.18, $n=6$; Hay *et al*., unpublished observations), but the affinity for $CGRP_{8-37}$ was clearly high. Unfortunately it was not possible to examine expression of CRLR or RAMPs in these cells. All clones of the Col-29 cells have originated from the same source but it seems that their phenotype with regard to CGRP pharmacology is particularly unstable. We have not been able to find any explanation for this behaviour nor have we succeeded in controlling it. It is clear that in the present study the cells are behaving pharmacologically in an identical manner to SK-N-MC cells and appear to express similar molecular components. Given that the cells can express CRLR and RAMPs, it is possible that an extra component may be responsible for modulating the $CGRP_{8-37}$ affinity to give a $CGRP_1$ or $CGRP_2$-like receptor. If this is the case, the expression of this unknown factor appears to be erratic.

Rat-2 fibroblasts bound adrenomedullin but not CGRP, as we have previously reported (Coppock *et al*., 1999). This correlated well with RAMP2 mRNA being present and RAMP1 mRNA being absent. CRLR mRNA levels were very low but an anti-rat CRLR antibody detected CRLR protein.

Despite the identical pharmacology of L6-1 and L6-2 cells (both CGRP and adrenomedullin receptors present), CRLR and RAMP mRNA composition between these cells was heterogenous. Whereas L6-2 cells had moderate levels of CRLR, RAMP1 and RAMP2 mRNA, we could not detect CRLR mRNA in L6-1 cells. RAMP1 and RAMP2 mRNA levels were much higher in L6-1 than in L6-2 cells. It is likely that CRLR mRNA in L6-1 cells was below our detection limit since CRLR was detected with the anti-rat CRLR antibody. The stability of the CRLR protein is not known and it is possible that a very small amount of CRLR mRNA gives rise to a very long half-life protein. However, the presence of both RAMP1 and RAMP2 mRNA corresponds

well with adrenomedullin and CGRP binding in both sources of L6 cells.

We did not detect RAMP3 mRNA in any cell line in this study. Either RAMP3 was not present or its levels were below our detection limits. Indeed we find RAMP3 mRNA to be expressed at very low levels in rat tissues (Chakravarty *et al*., 2000). At this time the precise function of RAMP3 is not known. It may be more important in forming amylin receptors with the calcitonin receptor (Tilakaratne *et al*., 2000; Muff *et al*., 1999) than adrenomedullin receptors *in vivo* or may have entirely different functions.

In conclusion, these results are in accordance with the CRLR/RAMP hypothesis proposed by Foord and co-workers. However there is an apparent mismatch between mRNA and protein data for CRLR in the L6-1 cells. This is likely to be due to very low levels of CRLR mRNA in these cells being below the detection limits of our system. Thus, circumstances where mRNA for CRLR is apparently absent by Northern analysis do not exclude this protein as the binding site for CGRP and/or adrenomedullin.

We thank the British Heart Foundation (Project grant PG/97091), the Wellcome Trust and Pfizer Ltd (Dr Claire Bihoreau, Pfizer Global Research and Development, Fresnes Laboratories, Fresnes, France) for supporting this work. Thanks also to Sue Kirkland for advice on the culture of Col-29 cells. D.L Hay is funded by a Medical Research Council studentship.

References

ABBOTT, C.R., ROSSI, M., KIM, M., ALAHMED, S.H., TAYLOR, G.M., GHATEI, M.A., SMITH, D.M. & BLOOM, S.R. (2000). Investigation of the melanocyte stimulating hormones on food intake. Lack of evidence to support a role for the melanocortin-3 receptor. *Brain Res.,* **869,** 203 – 210.

BHOGAL, R., SMITH, D.M., PURKISS, P. & BLOOM, S.R. (1993). Molecular identification of binding sites for calcitonin gene-related peptide (CGRP) and islet amyloid polypeptide (IAPP) in mammalian lung: species variation and binding of truncated CGRP and IAPP. *Endocrinology,* **133,** 2351 – 2361.

BRAIN, S.D. & CAMBRIDGE, H. (1996). Calcitonin gene-related peptide: vasoactive effects and potential therapeutic role. *Gen. Pharmacol.,* **27,** 607 – 611.

BUHLMANN, N., LEUTHAUSER, K., MUFF, R., FISCHER, J.A. & BORN, W. (1999). A receptor activity modifying protein (RAMP)2-dependent adrenomedullin receptor is a calcitonin gene-related peptide receptor when coexpressed with human RAMP1. *Endocrinology,* **140,** 2883 – 2890.

CARON, K.M. & SMITHIES, O. (2001). Extreme hydrops fetalis and cardiovascular abnormalities in mice lacking a functional adrenomedullin gene. *Proc. Natl. Acad. Sci.,* **98,** 615 – 619.

CHAKRAVARTY, P., SUTHAR, T.P., COPPOCK, H.A., NICHOLL, C.G., BLOOM, S.R., LEGON, S. & SMITH, D.M. (2000). CGRP and adrenomedullin binding correlates with transcript levels for calcitonin receptor-like receptor (CRLR) and receptor activity modifying proteins (RAMPs) in rat tissues. *Br. J. Pharmacol.,* **130,** 189 – 195.

CHIBA, T., YAMAGUCHI, A., YAMATANI, T., NAKAMURA, A., MORISHITA, T., INUI, T., FUKASE, M., NODA, T. & FUJITA, T. (1989). Calcitonin gene-related peptide receptor antagonist human CGRP-(8-37). *Am. J. Physiol.,* **256,** E331 – E335.

COPPOCK, H.A., OWJI, A.A., AUSTIN, C., UPTON, P.D., JACKSON, M.L., GARDINER, J.V., GHATEI, M.A., BLOOM, S.R. & SMITH, D.M. (1999). Rat-2 fibroblasts express specific adrenomedullin receptors, but not calcitonin-gene-related-peptide receptors, which mediate increased intracellular cAMP and inhibit mitogen-activated protein kinase activity. *Biochem. J.,* **338,** 15 – 22.

COPPOCK, H.A., OWJI, A.A., BLOOM, S.R. & SMITH, D.M. (1996). A rat skeletal muscle cell line (L6) expresses specific adrenomedullin binding sites but activates adenylate cyclase via calcitonin gene-related peptide receptors. *Biochem. J.,* **318,** 241 – 245.

COX, H.M. & TOUGH, I.R. (1994). Calcitonin gene-related peptide receptors in human gastrointestinal epithelia. *Br. J. Pharmacol.,* **113,** 1243 – 1248.

DENNIS, T., FOURNIER, A., CADIEUX, A., POMERLEAU, F., JOLICOEUR, F.B., ST. PIERRE, S. & QUIRION, R. (1990). hCGRP8-37, a calcitonin gene-related peptide antagonist revealing calcitonin gene-related peptide receptor heterogeneity in brain and periphery. *J. Pharmacol. Exp. Ther.,* **254,** 123 – 128.

DENNIS, T., FOURNIER, A., ST. PIERRE, S. & QUIRION, R. (1989). Structure-activity profile of calcitonin gene-related peptide in peripheral and brain tissues. Evidence for receptor multiplicity. *J. Pharmacol. Exp. Ther.,* **251,** 718 – 725.

DOODS, H. (2001). Development of CGRP antagonists for the treatment of migraine. *Curr. Opin. Investig. Drugs,* **2,** 1261 – 1268.

DUMONT, Y., FOURNIER, A., ST. PIERRE, S. & QUIRION, R. (1997). A potent and selective CGRP2 agonist, [Cys(Et)2,7]hCGRP alpha: comparison in prototypical CGRP1 and CGRP2 in vitro bioassays. *Can. J. Physiol. Pharmacol.,* **75,** 671 – 676.

EDVINSSON, L. (2001). Aspects on the pathophysiology of migraine and cluster headache. *Pharmacol. Tox.,* **89,** 65 – 73.

FINDLAY, D.M., HOUSSAMI, S., LIN, H.Y., MYERS, D.E., BRADY, C.L., DARCY, P.K., IKEDA, K., MARTIN, T.J. & SEXTON, P.M. (1994). Truncation of the porcine calcitonin receptor cytoplasmic tail inhibits internalization and signal transduction but increases receptor affinity. *Mol. Endocrinol.,* **8,** 1691 – 1700.

FLUHMANN, B., MUFF, R., HUNZIKER, W., FISCHER, R.A. & BON, W. (1995). A human orphan calcitonin receptor-like structure. *Biochem. Biophys. Res, Commun.,* **206,** 341 – 347.

HAGNER, S., HABERBERGER, R., KUMMER, W., SPRINGER, J., FISCHER, A., BOHM, S., GOKE, B. & MCGREGOR, G.P. (2001). Immunohistochemical detection of calcitonin gene-related peptide receptor (CGRPR-1) in the endothelium of human coronary artery and bronchial blood vessels. *Neuropeptides,* **35,** 1 – 7.

HAN, Z.Q., COPPOCK, H.A., SMITH, D.M., VAN NOORDEN, S., MAKGOBA, M.W., NICHOLL, C.G. & LEGON, S. (1997). The interaction of CGRP and adrenomedullin with a receptor expressed in the rat pulmonary vascular endothelium. *J. Mol. Endocrinol.,* **18,** 267 – 272.

HAY, D.L. & SMITH, D.M. (2001). Knockouts and transgenics confirm the importance of adrenomedullin in the vasculature. *Trends Pharmacol. Sci.,* **22,** 57 – 59.

HENKE, H., SIGRIST, S., LANG, W., SCHNEIDER, J. & FISCHER, J.A. (1987). Comparison of binding sites for the calcitonin gene-related peptides I and II in man. *Brain Res.,* **410,** 404 – 408.

HILAIRET, S., FOORD, S.M., MARSHALL, F.H. & BOUVIER, M. (2001). Protein-protein interaction and not glycosylation determines the binding selectivity of heterodimers between the calcitonin receptor-like receptor and the receptor activity-modifying proteins. *J. Biol. Chem.,* **31,** 29575 – 29581.

HINSON, J.P., KAPAS, S. & SMITH, D.M. (2000). Adrenomedullin, a multifunctional regulatory peptide. *Endocr. Rev.,* **21,** 138 – 167.

KAMITANI, S., ASAKAWA, M., SHIMEKAKE, Y., KUWASAKO, K., NAKAHARA, K. & SAKATA, T. (1999). The RAMP2/CRLR complex is a functional adrenomedullin receptor in human endothelial and vascular smooth muscle cells. *FEBS Lett.,* **448,** 111 – 114.

KAPAS, S., CATT, K.J. & CLARK, A.J. (1995). Cloning and expression of cDNA encoding a rat adrenomedullin receptor. *J. Biol. Chem.,* **270,** 25344 – 25347.

KAPAS, S. & CLARK, A.J. (1995). Identification of an orphan receptor gene as a type 1 calcitonin gene-related peptide receptor. *Biochem. Biophys. Res. Commun.,* **217,** 832 – 838.

KENNEDY, S.P., SUN, D., OLEYNEK, J.J., HOTH, C.F., KONG, J. & HILL, R.J. (1998). Expression of the rat adrenomedullin receptor or a putative human adrenomedullin receptor does not correlate with adrenomedullin binding or functional response. *Biochem. Biophys. Res. Commun.,* **244,** 832–837.

KIRKLAND, S.C. (1985). Dome formation by a human colonic adenocarcinoma cell line (HCA-7). *Cancer Res.,* **45,** 3790–3795.

LIN, H.Y., HARRIS, T.L., FLANNERY, M.S., ARUFFO, A., KAJI, E.H., GORN, A., KOLAKOWSKILF, J., LODISH, H.F. & GOLDRING, S.R. (1991). Expression cloning of an adenylate cyclase-coupled calcitonin receptor. *Science,* **254,** 1022–1024.

LONGMORE, J., HOGG, J.E., HUTSON, P.H. & HILL, R.G. (1994). Effects of two truncated forms of human calcitonin-gene related peptide: implications for receptor classification. *Eur. J. Pharmacol.,* **265,** 53–59.

MCLATCHIE, L.M., FRASER, N.J., MAIN, M.J., WISE, A., BROWN, J., THOMPSON, N., SOLARI, R., LEE, M.G. & FOORD, S.M. (1998). RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature,* **33,** 333–339.

MAKINO, I., HONDA, K., MAKINO, Y., OKANO, I., KANGAWA, K., KAMIYAHO, H.O., SHIBATA, K. & KAWARABAYASHI, T. (2001). Phenotypic changes of adrenomedullin receptor components, RAMP2 and CRLR mRNA expression in cultured rat vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.,* **258,** 515–520.

MORGAN, D.G., KULKARNI, R.N.,. HURLEY, J.D., WANG, Z.L., WANG, R.M., GHATEI, M.A., KARLSEN, A.E., BLOOM, S.R. & SMITH, D.M. (1998). Inhibition of glucose stimulated insulin secretion by neuropeptide Y is mediated via the Y1 receptor and inhibition of adenylyl cyclase in RIN 5AH rat insulinoma cells. *Diabetologia,* **41,** 1482–1491.

MUFF, R., BUHLMANN, N., FISCHER, J.A. & BORN, W. (1999). An amylin receptor is revealed following co-transfection of a calcitonin receptor with receptor activity modifying proteins-1 or -3. *Endocrinology,* **140,** 2924–2927.

MUFF, R., LEUTHAUSER, K., BUHLMANN, N., FOORD, S.M., FISCHER, J.A. & BORN, W. (1998). Receptor activity modifying proteins regulate the activity of a calcitonin gene-related peptide receptor in rabbit aortic endothelial cells. *FEBS Lett.,* **441,** 366–368.

NIKITENKO, L.L., BROWN, N.S., SMITH, D.M., MACKENZIE, I.Z., BICKNELL, R. & RESS, M.C.P. (2001). Differential and cell-specific expression of calcitonin receptor-like receptor and receptor activity modifying proteins in the human uterus. *Mol. Human Reproduction,* **7,** 655–664.

NJUKI, F., NICHOLL, C.G., HOWARD, A., MAK, J.C., BARNES, P.J., GIRGIS, S.I. & LEGON, S. (1993). A new calcitonin-receptor-like sequence in rat pulmonary blood vessels. *Clin. Sci. Colch.,* **85,** 385–388.

OLIVER, K.R., KANE, S.A., SALVATORE, C.A., MALLEE, J.J., KINSEY, A.M., KOBLAN, K.S., KEYVAN-FOULADI, N., HEAVENS, R.P., WAINWRIGHT, A., JACOBSON, M., DICKERSON, I.M. & HILL, R.G. (2001). Cloning, characterization and central nervous system distribution of receptor activity modifying proteins in the rat. *Eur. J. Neuroscience,* **14,** 618–628.

OWJI, A.A., SMITH, D.M., COPPOCK, H.A., MORGAN, D.G., BHOGAL, R., GHATEI, M.A. & BLOOM, S.R. (1995). An abundant and specific binding site for the novel vasodilator adrenomedullin in the rat. *Endocrinology,* **136,** 2127–2134.

POYNER, D.R., ANDREW, D.P., BROWN, D., BOSE, C. & HANLEY, M.R. (1992). Pharmacological characterization of a receptor for calcitonin gene-related peptide on rat, L6 myocytes. *Br. J. Pharmacol.,* **105,** 441–447.

POYNER, D.R., SEXTON, P.M., MARSHALL, I., SMITH, D.M., QUIRION, R., BORN, W., MUFF, R., FISCHER, J.A. & FOORD, S.M. (2002). International Union of Pharmacology. The mammalian CGRP, adrenomedullin, amylin and calcitonin receptors. *Pharmacol Rev.,* **54,** 1–14.

POYNER, D.R., SOOMETS, U., HOWITT, S.G. & LANGEL, U. (1998). Structural determinants for binding to CGRP receptors expressed by human SK-N-MC and Col-29 cells: studies with chimeric and other peptides. *Br. J. Pharmacol.,* **124,** 1659–1666.

RORABAUGH, B.R., SCOFIELD, M.A., SMITH, D.D., JEFFRIES, W.B. & ABEL, P.W. (2001). Functional calcitonin gene-related peptide subtype 2 receptors in porcine coronary arteries are identified as calcitonin gene-related peptide subtype 1 receptors by radioligand binding and reverse transcription-polymerase chain reaction. *J. Pharmacol. Exp. Ther.,* **299,** 1086–1094.

SEXTON, P.M., MCKENZIE, J.S., MASON, R.T., MOSELEY, J.M., MARTIN, T.J. & MENDELSOHN, F.A. (1986). Localization of binding sites for calcitonin gene-related peptide in rat brain by in vitro autoradiography. *Neuroscience,* **19,** 1235–1245.

SHARMA, S.K., AUSTIN, C., HOWARD, A., LO, G., NICHOLL, C.G. & LEGON, S. (1992). Characterization of rat gastric inhibitory peptide cDNA. *J. Mol. Endocrinol.,* **9,** 265–272.

SULISTIYANI & ST. CLAIR, R.W. (1991). The method of isolation of primary cells and their subculture influences the expression of LDL receptors on pigeon and chick embryo cells in culture. *Atherosclerosis,* **91,** 123–135.

TILAKARATNE, N., CHRISTOPOULOS, G., ZUMPE, E.T., FOORD, S.M. & SEXTON, P.M. (2000). Amylin receptor phenotypes derived from human calcitonin receptor/RAMP coexpression exhibit pharmacological differences dependent on receptor isoform and host cell environment. *J. Pharmacol. Exp. Ther.,* **294,** 61–72.

UEDA, T., UGAWA, S., SAISHIN, Y. & SHIMADA, S. (2001). Expression of receptor-activity modifying protein (RAMP) mRNAs in the mouse brain. *Mol. Brain Res.,* **93,** 36–45.

UPTON, P.D., AUSTIN, C., TAYLOR, G.M., NANDHA, K.A., CLARK, A.J., GHATEI, M.A., BLOOM, S.R. & SMITH, D.M. (1997). Expression of adrenomedullin (ADM) and its binding sites in the rat uterus: increased numbers of binding sites and ADM messenger ribonucleic acid in 20-day old pregnant rats compared with non pregnant rats. *Endocrinology,* **138,** 2508–2514.

WISSKIRCHEN, F.M., BURT, R.P. & MARSHALL, I. (1998). Pharmacological characterization of CGRP receptors mediating relaxation of the rat pulmonary artery and inhibition of twitch responses of the rat vas deferens. *Br. J. Pharmacol.,* **123,** 1673–1683.

WU, D., EBERLEIN, W., RUDOLF, K., ENGEL, W., HALLERMAYER, G. & DOODS, H. (2000). Characterisation of calcitonin gene-related peptide receptors in rat atrium and vas deferens: evidence for a [Cys(Et)(2,7)]hCGRP-preferring receptor. *Eur. J. Pharmacol.,* **400,** 313–319.

(Received January 3, 2002
Revised March 25, 2002
Accepted April 15, 2002)