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Characterization of receptors for calcitonin gene-related peptide and adrenomedullin on the guinea-pig vas deferens

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1 The receptors which mediate the effects of calcitonin gene-related peptide (CGRP), amylin and adrenomedullin on the guinea-pig vas deferens have been investigated.

2 All three peptides cause concentration dependant inhibitions of the electrically stimulated twitch response (pD_2s for CGRP, amylin and adrenomedullin of 7.90 ± 0.11, 7.70 ± 0.19 and 7.25 ± 0.10 respectively).

3 $CGRP_{8-37}$ (1 μM) and AC187 (10 μM) showed little antagonist activity against adrenomedullin.

4 $Adrenomedullin_{22-52}$ by itself inhibited the electrically stimulated contractions of the vas deferens and also antagonized the responses to CGRP, amylin and adrenomedullin.

5 [^{125}I]-adrenomedullin labelled a single population of binding sites in vas deferens membranes with a pIC_{50} of 8.91 and a capacity of 643 fmol mg^{-1}. Its selectivity profile was adrenomedullin > AC187 > CGRP = amylin. It was clearly distinct from a site labelled by [^{125}I]-CGRP ($pIC_{50} = 8.73$, capacity = 114 fmol mg^{-1}, selectivity CGRP > amylin = AC187 > adrenomedullin). [^{125}I]-amylin bound to two sites with a total capacity of 882 fmol mg^{-1}.

6 Although CGRP has been shown to act at a $CGRP_2$ receptor on the vas deferens with low sensitivity to $CGRP_{8-37}$, this antagonist displaced [^{125}I]-CGRP with high affinity from vas deferens membranes. This affinity was unaltered by increasing the temperature from 4°C to 25°C, suggesting the anomalous behaviour of $CGRP_{8-37}$ is not due to temperature differences between binding and functional assays.

Keywords: CGRP; amylin; adrenomedullin; $CGRP_2$ receptor; vas deferens, guinea-pig; $adrenomedullin_{22-52}$; $CGRP_{8-37}$

Abbreviations: CGRP, calcitonin gene-related peptide; CRLR, calcitonin receptor-like receptor; RAMP, receptor activity modifying protein

Introduction

Adrenomedullin is a peptide which was recently isolated from human phaeochromocytomas (Kitamura *et al*., 1993a). Human and rat adrenomedullin are respectively 52 and 50 amino acids long. It is possible to remove the first 12 amino acids with little change in potency (Lin *et al*., 1994). The resulting peptide shows some similarity to members of the calcitonin family of peptides, especially CGRP and amylin. Although homology at the level of the primary sequence is weak between adrenomedullin and either CGRP or amylin, there are stronger relationships between the secondary structures of the peptides. They all have a six residue ring structure close to their N-termini, formed by an intramolecular disulphide bond. This is then followed by a region of potential amphipathic α-helix, and they all have a C-terminal amide (Kitamura *et al*., 1993b).

In view of the similarity between CGRP, adrenomedullin and amylin, it might be expected that there would be cross-reactivity between these peptides at their cognate receptors. The pharmacology of these peptides is complicated, but recent data suggests that the CGRP and adrenomedullin receptors may be the product of the same gene, called calcitonin receptor like receptor (CRLR) (Aiyar *et al*., 1996). Specificity is determined by accessory proteins belonging to the RAMP family (receptor activity modifying proteins), with RAMP1 co-expression leading to a CGRP receptor, and RAMP2 co-expression resulting in an adrenomedullin receptor (McLachtie *et al*., 1998). There is also evidence for subtypes of CGRP receptors (Quirion *et al*., 1992, Poyner, 1995; 1997b for reviews). To a first approximation CGRP receptors can be divided into two subclasses; $CGRP_1$ which have a high affinity for the antagonist $CGRP_{8-37}$ and $CGRP_2$, which are much less sensitive to this compound. CRLR gives a $CGRP_1$-like receptor. Both amylin and adrenomedullin can activate $CGRP_1$ receptors, although they are less potent than CGRP itself. Little is known about the potencies of these peptides at $CGRP_2$ receptors and relatively little characterization of any kind has been done on $CGRP_2$ receptors. One major puzzle with this subclass of receptors is that $CGRP_{8-37}$ shows a high affinity in radioligand binding studies, in contrast to its behaviour in functional studies (Dennis *et al*., 1990; Quirion *et al*., 1992). There is no explanation for this discrepancy, although it has been noted that the binding and functional assays are carried out under very different conditions (Poyner, 1995).

Amylin can bind to a site that has a very distinctive pharmacology, having a high affinity for calcitonin and somewhat lower affinity for CGRP (Veale *et al*., 1994). This site was first discovered in the CNS and is often termed the C3

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binding site (Sexton *et al*., 1988). It is likely to represent a receptor through which amylin mediates many of its unique biological effects (Beaumont *et al*., 1995). There is evidence to suggest that this receptor may be a version of the C1a calcitonin receptor which has been subject to post-translational modification, or association with another protein such as RAMP1 (Chen *et al*., 1997; Sexton *et al*., 1999). Amylin also has a second class of binding site which is found in peripheral tissues; however this has a higher affinity for adrenomedullin and probably represents an adrenomedullin receptor (Aiyar *et al*., 1995; Owji *et al*., 1995). Radio-iodinated adrenomedullin binds to sites which have extremely high specificity for adrenomedullin over CGRP and amylin. It is possible that these correspond to the sites which are labelled with amylin and their apparent extreme specificity is an artefact due to the iodination of adrenomedullin (Owji *et al*., 1995), although functional studies of adrenomedullin receptors in Rat2 and Swiss 3T3 cells do confirm that these receptors are only very poorly activated by CGRP (Withers *et al*., 1996; D.M.S. & H.A. Coppock, unpublished observations).

Adrenomedullin is a very potent vasodilator, a property which it shares with CGRP. However, it is widely distributed, apparently functioning as a local hormone in many tissues, and it is apparent that its actions extend beyond the cardiovascular system. We have previously reported the existence of distinct receptors that mediate the effects of CGRP and amylin on the guinea-pig vas deferens (Tomlinson & Poyner, 1996). In this report we extend these studies to show the existence of a separate adrenomedullin receptor on this tissue. We also have further characterized the $CGRP_2$ receptor subtype which is found here.

Some of these results have been previously published in abstract form (Poyner, 1997a).

Methods

Materials

Rat adrenomedullin was obtained from Peptide Institute Inc. (Osaka, Japan) and human αCGRP was obtained from Calbiochem (Nottingham, U.K.). Rat αCGRP and amylin were custom synthesized by ASG University (Szedgel, Hungary). It should be noted that rat and human CGRP show essentially identical properties in binding to and activating CGRP receptors (see Poyner, 1992 for review). All peptides were checked for correct molecular weight by mass spectroscopy. Peptide solutions were made up as 1 mM or 100 μM stock solutions in distilled water based on their nominal molecular weight, and subsequently diluted in buffer containing 0.1% bovine serum albumin. [^{125}I]-iodohistidyl human αCGRP (specific activity 74TBq mmol^{-1}) and Na [^{125}I] were supplied by Amersham International (Little Chalfont, Bucks, U.K.). [^{125}I]-Bolton Hunter reagent was supplied by NEN Life Science Products (Hounslow, Middlesex, U.K.). Iodogen reagent was supplied by Pierce (Rockford, Illinois, U.S.A.). Other reagents were obtained as described previously (Tomlinson & Poyner, 1996).

Peptide iodination

Rat adrenomedullin was iodinated by the iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) method as previously described (Owji *et al*., 1995). Briefly, 12.5 μg (2 nmoles) of peptide in 10 μl of 0.2 M phosphate buffer pH 7.2 were reacted with 37 MBq of Na [^{125}I] and 10 μg of iodogen reagent for 4 min at 22°C. The [^{125}I]-peptide was purified by reversed-phase high performance liquid chromatography (Waters C_{18} Novapak, Millipore, Milford MA, U.S.A.) using a 15–40% acetonitrile/water/0.05% trifluoroacetic acid gradient. Fractions showing binding were aliquoted, freeze-dried and stored at −80°C. The specific activity was 10 Bq fmol^{-1}, as determined by radio-immunoassay.

Rat amylin was iodinated by the Bolton-Hunter method as previously reported (Bhogal *et al*., 1992) except that the reaction was performed at pH 9 for 1 h at room temperature. Its specific activity was 33 Bq fmol^{-1}, determined by comparison of saturation and competitive binding assays.

Membrane preparation

Membranes were prepared by differential centrifugation as previously described (Bhogal *et al*., 1993). Briefly, whole vas deferens from Dunkin-Hartley guinea-pigs (350 g, Harlan U.K. Ltd, Bicester, Oxon, U.K.) were homogenized in ice-cold 50 mM HEPES pH 7.6 containing 0.25 M sucrose, 10 μg ml^{-1} pepstatin, 0.25 μg ml^{-1} leupeptin and antipain, 0.1 mg ml^{-1} benzamidine and bacitracin and 30 μg ml^{-1} aprotinin. The homogenates were centrifuged at 1500 × g for 20 min at 4°C and the supernatants centrifuged at 100,000 × g for 1 h at 4°C. The pellets were resuspended in 10 volumes of the above buffer without sucrose and centrifuged at 100,000 × g for 1 h at 4°C.

Adrenomedullin binding assays

Membranes (100 μg protein) were incubated for 30 min at 4°C in 0.5 ml of binding buffer of composition (mM); HEPES, 20 (pH 7.4); $MgCl_2$, 5; NaCl, 10; KCl, 4; EDTA, 1; phosphoramidon, 0.001 and 0.3% bovine serum albumin containing 500 Bq (100 pM) [^{125}I]-adrenomedullin (Owji *et al*., 1995). Bound and free label was separated by centrifugation at 15,000 × g for 2 min at 4°C. Non specific binding was determined in the presence of 1 μM unlabelled rat adrenomedullin. The integrity of the labels after binding was determined by fast protein liquid chromatography using reversed-phase C_2/C_{18} columns (pepRPC HR5/5, Pharmacia Biotech, St Albans, Herts, U.K.) as previously described (Bhogal *et al*., 1992) and more than 95% co-eluted with the standard (results not shown).

Amylin binding assays

Vas deferens membranes (100 μg) were incubated for 60 min at 4°C with 500 Bq (30 pM) [^{125}I]-rat amylin in binding buffer as for the adrenomedullin binding assays. Bound and free label were separated by centrifugation as described above and non-specific binding was determined in the presence of 200 nM rat amylin.

CGRP binding assays

Vas deferens membranes (100 μg) were resuspended in 0.5 ml of buffer of the following composition (mM): sodium chloride, 100; bacitracin, 0.4; HEPES 20 (pH 7.5) and 0.3% bovine serum albumin. Binding assays were carried out in the presence of 100 pM [^{125}I]-iodohistidyl-human αCGRP for either 30 min (at 25°C) or 2 h (at 4°C). Assays were terminated by microcentrifugation, as described above. Non-specific binding was determined in the presence of 1 μM CGRP.

Protein measurements

Protein was measured by the Biuret assay.

Contractile responses of the isolated vas deferens

Vas deferens were removed from Dunkin-Hartley guinea-pigs and dose-response curves to peptides were obtained as described previously (Tomlinson & Poyner, 1996).

Analysis of data

Binding data from the [^{125}I]-adrenomedullin or amylin displacement experiments was analysed by non-linear regression using the 'Receptor-Fit' programme (Lundon Software, Cleveland, OH, U.S.A.) to calculate the dissociation constant (K_D), the IC_{50} and the concentration of binding sites (B_{max}). Analysis of one site versus two site competition curves was by F-test, with two component fits considered significant at $P<0.05$. The analysis of the [^{125}I]-CGRP displacement curves and concentration response curves was done with EBDA/LIGAND to estimate IC_{50}/EC_{50} values and Hill coefficients (n_H). Analysis of Hill coefficients was by Students unpaired *t*-test, with a site being considered heterogeneous if the coefficient was significantly different ($P<0.05$) from unity.

The IC_{50}/EC_{50} values from individual experiments (expressed in terms of molarity) were converted to pIC_{50}s by taking their negative logarithim. They are expressed as means$\pm$s.e.means throughout the text.

For the contractile studies, each dose response curves was fitted to a logistic equation by means of the EBDA/Ligand routine to obtain an EC_{50} for the agonist. These were converted to pD_2 values by taking negative logarithims. Responses were expressed as percentages of the resting tension before addition of agonist (but after addition of antagonist). The effects of antagonists were examined by comparing responses before and after antagonist treatment by means of Student's unpaired *t*-test or a one-way ANOVA followed by Dunnett's test depending on whether single or multiple comparisons were being made. Significance was accepted at the $P<0.05$ level.

Results

Contractile responses of the isolated vas deferens

In confirmation of previous results (Tomlinson & Poyner, 1996), both human αCGRP and rat amylin produced dose-dependent inhibitions of the contractions of the electrically stimulated guinea-pig vas deferens (pD_2s of 7.90$\pm$0.11 and 7.70$\pm$0.19 respectively; Figure 1). Adrenomedullin also caused a potent inhibition of electrically stimulated contractions, producing an effect very similar to that of amylin (pD_2 of 7.25$\pm$0.10; Figure 1a). Previously it was shown that the responses to both CGRP and amylin were insensitive to $CGRP_{8-37}$, but the response to CGRP was inhibited by AC187. The response to adrenomedullin was completely unaltered by the presence of 1 μM $CGRP_{8-37}$. In the presence of 10 μM AC187 there was a small rightwards shift (pD_2 of 6.55$\pm$0.24), which however failed to achieve significance (Figure 1).

There have been reports that $adrenomedullin_{22-52}$ can block some effects of adrenomedullin. When added to this preparation it was obvious that the chief response of $adrenomedullin_{22-52}$ was itself to reduce the magnitude of the electrically stimulated contraction (Figure 2). Neither $CGRP_{8-37}$ nor AC187 altered resting tension. In addition to this effect it also significantly inhibited the response to adrenomedullin, but this was also seen against CGRP and amylin (Figure 1a–c), although the inhibition of the amylin response was much less marked than that seen against the other two peptides.

Radioligand binding studies with [^{125}I]-adrenomedullin

[^{125}I]-adrenomedullin bound with high affinity to a single class of binding sites on membranes made from guinea-pig vas deferens (Table 1, Figure 3a), with a capacity of 646$\pm$34 fmol receptor mg^{-1} protein. CGRP and amylin were both well over a hundred times less potent at displacing the radiolabel than adrenomedullin itself. AC187 was the most potent inhibitor of [^{125}I]-adrenomedullin binding, but even this was 75 fold less potent than adrenomedullin (Table 1, Figure 3b).

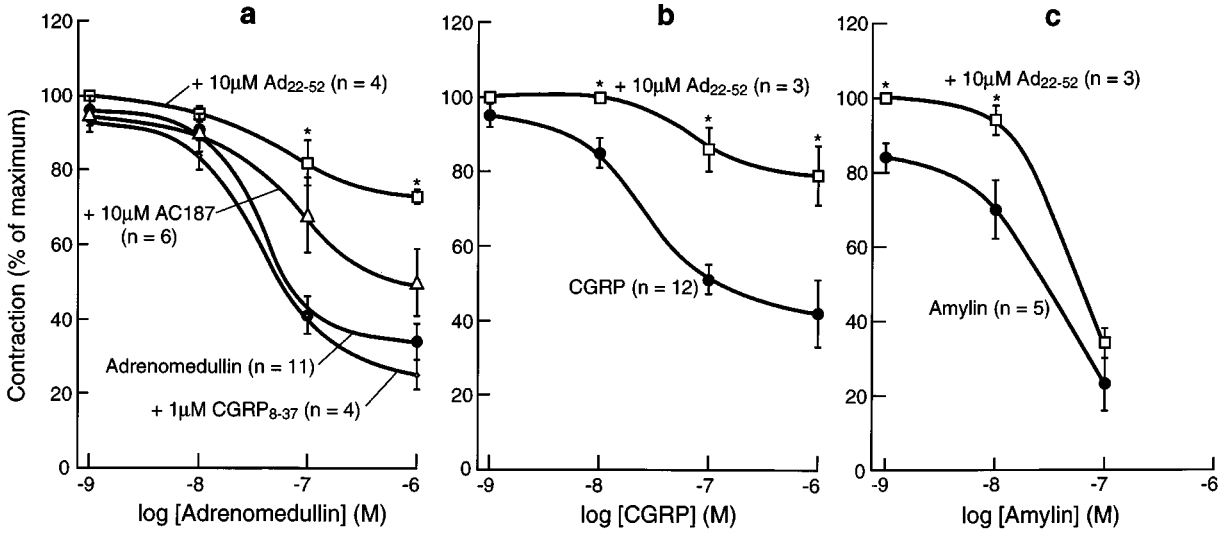

Figure 1 Relaxation of the electrically stimulated vas deferens by (a) adrenomedullin, (b) CGRP and (c) amylin in the presence of potential antagonists. Values are means$\pm$s.e.means, *n* values shown in brackets. *Significantly different from response to agonist alone, $P<0.05$.

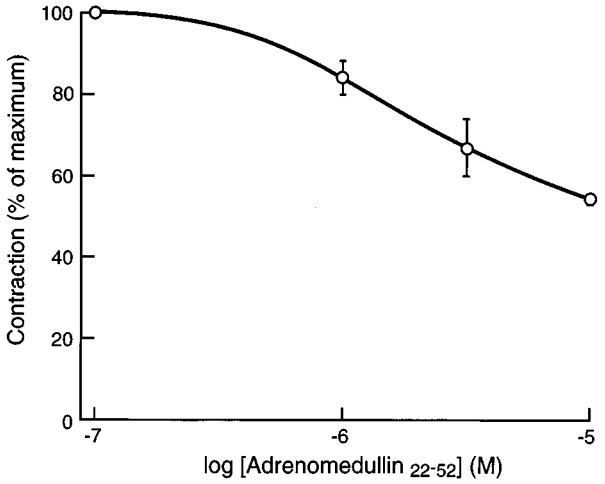

Figure 2 Relaxation of the electrically stimulated vas deferens by adrenomedullin$_{22-52}$, Values are means±s.e.means of three determinations.

Table 1 pIC_{50} values for ligands binding to guinea-pig vas deferens membranes

	[^{125}I]-adrenomedullin	*[^{125}I]-CGRP*	*[^{125}I]-amylin*
Adrenomedullin	8.91±0.09 (4)	6.89±0.21 (3)	7.84±0.13 (3)
CGRP	6.65±0.11 (3)	8.73±0.12 (3)	nd
Amylin	6.39±0.14 (3)	7.87±0.28 (4)	8.53±0.20* (3)
AC187	7.03±0.18 (3)	7.58±0.20 (3)	nd
$CGRP_{8-37}$	nd	8.78±0.11 (4)	nd

nd = not determined. Values are means±s.e.means; *n* values are shown in brackets. *pIC_{50} values for a 2-site fit are 9.78 and 7.88.

Radioligand binding studies with [^{125}I]-amylin

[^{125}I]-amylin bound to sites with a total capacity of 882±172 fmol receptor mg^{-1} protein (Figure 4). The binding curve was best fitted to a two-site model, where the high affinity site had an IC_{50} of 0.17 nM and the low affinity site had an IC_{50} of 12.9 nM Adrenomedullin did not discriminate between these two sites in displacement studies, and had an IC_{50} of 14.5 nM.

Radioligand binding studies with [^{125}I]-CGRP

[^{125}I]-CGRP bound with high affinity to a single class of sites (n_H = 0.77±0.17, *n* = 3) on vas deferens with a capacity of 114 fmol receptor mg^{-1} protein (Table 1). In agreement with previous studies, $CGRP_{8-37}$ was a potent inhibitor of this binding, in spite of being a very weak antagonist in functional studies. Rat amylin and AC187 were over an order of magnitude less potent than CGRP, and adrenomedullin was about two orders of magnitude less potent than CGRP (Table 1, Figure 5a).

In order to investigate further the anomalous behaviour of $CGRP_{8-37}$ binding, the temperature dependency of its binding was investigated. The above experiments were carried out at 4°C, following published procedures (Dennis *et al.*, 1989). However, the functional assays were carried out at 37°C. It proved difficult to obtain reproducible binding data at 37°C, but binding was possible at 24°C (Figure 5b and c). For $CGRP_{8-37}$ pIC_{50}/n_H values were 8.78±0.11/0.77±0.17 at 4°C and 8.50±0.13/1.31±0.21 at 24°C. For CGRP itself, pIC_{50}/n_H values were 8.73±0.12/0.89±0.20 at 4°C and 8.62±0.13/0.77±0.20 at 24°C. Thus there were no substantial changes to the affinities of either CGRP or $CGRP_{8-37}$, implying that temperature is not an important factor in modulating ligand-receptor interactions at the $CGRP_2$ receptor 'subtype'.

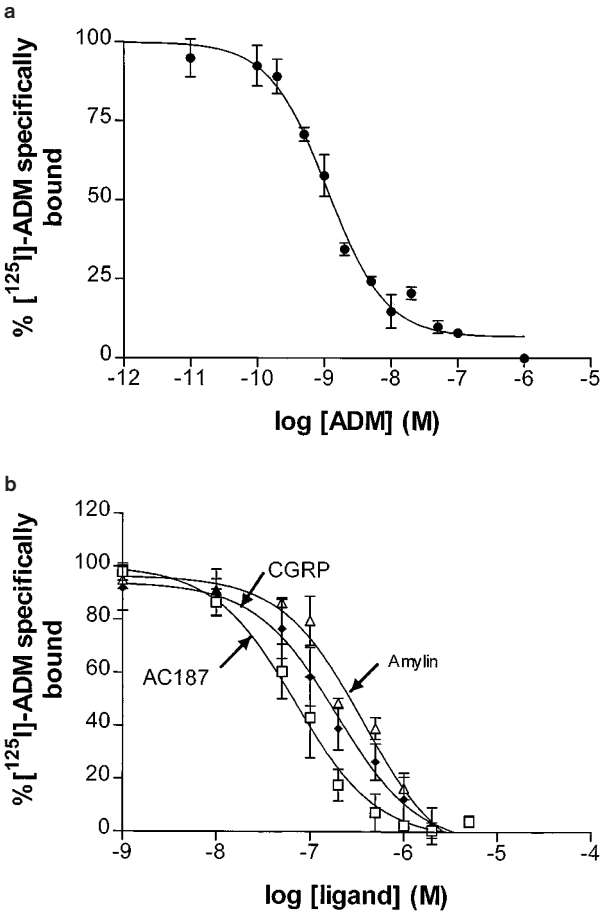

Figure 3 Displacement of [^{125}I]-adrenomedullin by (a) adrenomedullin and (b) AC187, CGRP and amylin in vas deferens membranes. Values are means±s.e.means of three determinations.

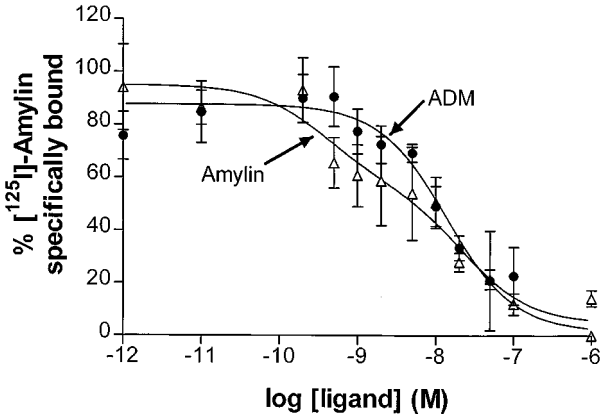

Figure 4 Displacement of [^{125}I]-amylin by amylin and adrenomedullin in vas deferens membranes. Values are means±s.e.means of three determinations.

Discussion

These results demonstrate the presence of an adrenomedullin receptor on the guinea-pig vas deferens. This paper also

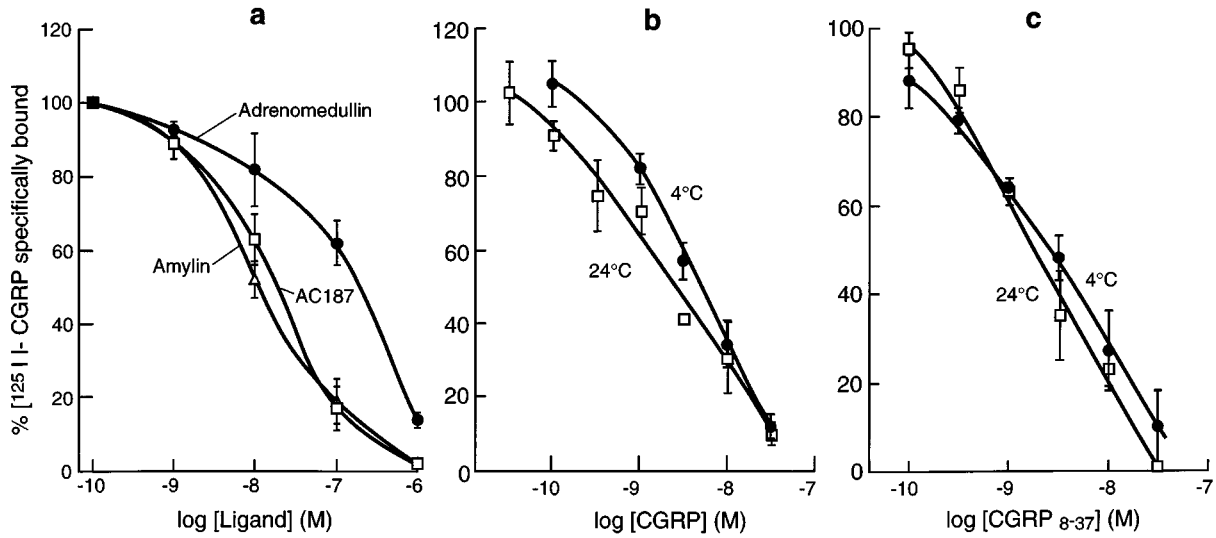

Figure 5 Displacement of [^{125}I]-CGRP in vas deferens membranes. (a) Displacement by adrenomedullin, AC187 and amylin. Effects of temperature on the displacement by (b) CGRP and (c) $CGRP_{8-37}$. Values are means ± s.e.means of 3–4 determinations.

presents new evidence as to the properties of the distinctive $CGRP_2$-type receptor found on this tissue.

Adrenomedullin inhibited electrically stimulated contractions of the vas deferens in a similar manner to CGRP. Further characterization of this response by functional assays was inconclusive. $CGRP_{8-37}$ failed to block the adrenomedullin response, but it would be expected to be inactive at both $CGRP_2$ and adrenomedullin receptors. $Adrenomedullin_{22-52}$ has been reported selectively to antagonize adrenomedullin in some tissues e.g. NG108-15 neuroblastoma cells (Zimmermann *et al*., 1996). However, *in-vivo* it seems much less effective (Champion *et al*., 1997; Santiago *et al*., 1995). In the vas deferens it was active by itself in inhibiting electrically stimulated contractions, and whilst it could additionally antagonize the response to adrenomedullin, it had very similar effects on the responses to CGRP and to some extent, amylin. Whilst inspection of Figure 1 suggests that $adrenomedullin_{22-52}$ might be acting non-competitively against CGRP and adrenomedullin, it is difficult to be certain that a true maximum to those two peptides had been established in the presence of the antagonist. AC187 was also inconclusive. This was previously reported to have no effect on the response to CGRP but to block the actions of amylin. This caused a small but non-significant shift in the concentration-response curve to adrenomedullin, but it was not possible to use it at higher concentrations to confirm this response. Broadly similar results have been observed on Rat 2 cells, where AC187 was only a weak antagonist at 10 μM against adrenomedullin (D.M.S. & H.A. Coppock, unpublished observations).

Radioligand binding did yield more conclusive data. It was possible to measure specific binding to both CGRP and adrenomedullin in the vas deferens. However these yielded very different results. The binding site labelled by [^{125}I]-adrenomedullin bound CGRP and amylin with over 200 fold lower affinities compared to adrenomedullin; the converse was found to be true for the binding site labelled with [^{125}I]-CGRP. The capacities of the two sites are also very different. Thus the binding data strongly suggests that CGRP and adrenomedullin interact with distinct sites in the vas deferens.

The adrenomedullin binding data resembles that previously observed with this radioligand where a number of studies have shown that CGRP is at least two orders of magnitude less potent than adrenomedullin (e.g. Coppock *et al*., 1996; Owji *et al*., 1995: Zimmermann *et al*., 1996; Upton *et al*., 1997). Little comparative work has been done on adrenomedullin and amylin, but what data does exist is again broadly in line with this study, showing that amylin is substantially less potent than adrenomedullin (Zimmermann *et al*., 1996; Owji *et al*., 1995). The affinity of AC187 is also in line with that found by radioligand binding on Rat 2 cells. The lack of a specific effect of $adrenomedullin_{22-52}$ in contrast to some other reports might argue for heterogeneity amongst adrenomedullin receptors. However, caution is needed, since the compound has only been used in comparatively few studies and the data in the present study indicates that it is important to determine its specificity if it does antagonize adrenomedullin.

The nature of the $CGRP_2$ receptor in the vas deferens is curious. Our data confirm what has previously been reported; that although $CGRP_{8-37}$ is a weak antagonist in functional studies on this tissue, it binds with high affinity in radioligand binding studies (Dennis *et al*., 1990; Quirion *et al*., 1992). In previous studies, the radioligand binding was carried out at 4°C in a low ionic strength buffer. As these conditions are very different from those used in functional studies, it was important to check whether or not they influenced the affinity for $CGRP_{8-37}$; temperature effects have been reported for the binding of this compound to $CGRP_1$ receptors expressed in rat L6 myocytes (Poyner *et al*., 1992). Clearly, the data in our study show that temperature or buffer composition are unlikely to play a significant role in determining the affinity of $CGRP_{8-37}$ to $CGRP_2$-type receptors. The anomaly could be an artefact caused by the iodination of CGRP, altering its selectivity. It may also be that some very labile accessory protein (lost during the course of membrane preparation) is responsible for promoting the low antagonist-affinity form of the $CGRP_2$ receptor. The recent description of RAMP which is required for expression of CGRP binding by CRLR may be an interesting precedent for this type of interaction (McLachtie *et al*., 1998). However, it should be stressed that the molecular nature of the $CGRP_2$ receptor is unknown.

A second interesting feature about the $CGRP_2$ receptor in the vas deferens is its selectivity for CGRP over other peptides; adrenomedullin in particular has a low affinity. This contrasts with a number of binding studies carried out on $CGRP_1$-like receptors. In rat L6 cells, human SK-N-MC cells and HEK293 cells permanently transfected with CRLR, the relative potency

order for displacement of [^{125}I]-CGRP binding is CGRP > adrenomedullin > amylin (Poyner *et al*., 1992; Coppock *et al*., 1996; Vine *et al*., 1996; Han *et al*., 1997). Further work is required to establish whether or not this might be a diagnostic feature for $CGRP_2$ receptors, but our preliminary data on human Col 29 cells which express a $CGRP_2$ receptor indicates that adrenomedullin has an EC_{50} at least 100 fold lower than that for CGRP. Of course, if the $CGRP_{8-37}$ affinity at this receptor as determined by radioligand binding does not match that measured in functional assays, it cannot be excluded that these other peptides may show a similar phenomenon. However, the concept that the $CGRP_2$ receptor shows pronounced selectivity for CGRP over other peptides is consistent with our functional data reported here and previously (Tomlinson & Poyner, 1996).

The receptor at which amylin exerts its action remains to be determined. The profile of the binding site labelled by [^{125}I]-amylin is distinct from that labelled by CGRP or adrenomedullin. Furthermore, amylin shows poor ability to displace both [^{125}I]-adrenomedullin and [^{125}I]-CGRP. In the contractile studies $adrenomedullin_{22-52}$ appeared to be less effective against amylin than adrenomedullin or CGRP. The simplest conclusion is that amylin has an independent receptor. The lack of effects of AC187 suggest that amylin is not acting at its C3 binding site. The peripheral amylin binding site in the lung has a higher affinity for adrenomedullin compared to amylin (and may in fact be identical with the adrenomedullin receptor of that tissue; Owji *et al*., 1995), but this does not fully match the binding profile found in this study. However, the issue is complicated by the presence of multiple binding sites for [^{125}I]-amylin. The question of which receptor or receptors amylin interacts with will probably only be solved once the sequences of these receptors become available.

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