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$CGRP_2$ receptor in the internal anal sphincter of the rat: implications for CGRP receptor classification

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1 The CGRP receptor mediating relaxation of the rat internal anal sphincter (IAS) has been characterized using CGRP analogues, homologues, the antagonist $CGRP_{8-37}$ and its analogues.

2 In isolated IAS strips, the spontaneously developed tone was concentration-dependently relaxed by hα CGRP, hβ CGRP and rat β CGRP (pEC_{50} 8.1±0.2, 8.3±0.1 and 8.4±0.2, respectively; 100% maximum response). Vasoactive intestinal polypeptide (VIP) was around 7 fold more potent than hα CGRP (pEC_{50} 9.0±0.1; 100% maximum relaxation). [Cys($ACM^{2,7}$)] hα CGRP and salmon calcitonin were inactive (up to 10^{-5} M).

3 Hα $CGRP_{8-37}$ (10^{-5} M) antagonized responses to hα CGRP (apparent pK_B 5.7±0.3) and rat β CGRP (apparent pK_B 5.8±0.2), but not to VIP. Hβ $CGRP_{8-37}$ (10^{-5} M) was an antagonist against hα CGRP (apparent pK_B 6.1±0.1). Hα $CGRP_{8-37}$ analogues (10^{-5} M), with substitutions at the N-terminus by either glycine[8] or *des*-NH_2 valine[8] or proline[8], antagonized hα CGRP responses with similar affinities (apparent pK_B 5.8±0.1, 5.8±0.1 and 5.5±0.1, respectively).

4 Peptidase inhibitors (amastatin, bestatin, captopril, phosphoramidon and thiorphan, 10^{-6} M each) did not increase the agonist potency of either hα CGRP or [Cys($ACM^{2,7}$)] hα CGRP, or the antagonist affinity of hα $CGRP_{8-37}$ against hα CGRP or rat β CGRP.

5 These data demonstrate for the first time a CGRP receptor in the rat IAS for which hα $CGRP_{8-37}$ and its analogues have an affinity that is consistent with a $CGRP_2$ receptor. However, there is a marked species difference as the antagonist has a 100 fold lower affinity in the rat than in the same tissue of the opossum (Chakder & Rattan, 1991).

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Keywords: hα CGRP; hβ CGRP; rat β CGRP; [Cys($ACM^{2,7}$)] hα CGRP; hα $CGRP_{8-37}$; hβ $CGRP_{8-37}$; peptidase inhibitors; rat internal anal sphincter; $CGRP_2$ receptor

Abbreviations: IAS, internal anal sphincter; TTX, tetrodotoxin; VIP, vasoactive intestinal polypeptide

Introduction

Receptors for CGRP have been divided into two subtypes, $CGRP_1$ and $CGRP_2$ (Dennis *et al*., 1989; 1990; Mimeault *et al*., 1991; Quirion *et al*., 1992), largely based on the differing antagonist affinities for hα $CGRP_{8-37}$. This is significantly more potent at the $CGRP_1$ receptor in the guinea-pig atrium (pA_2 7.2–7.7) than at the $CGRP_2$ receptor in the rat vas deferens (pA_2 6.2; Mimeault *et al*., 1991).

One complication in the classification of CGRP receptors may be that it relies on comparisons between guinea-pig and rat tissues. Studies in the rat have now shown that hα $CGRP_{8-37}$ displays different affinities between the pulmonary artery (pA_2 6.9) and the vas deferens (pA_2 6.0), supporting the characterization of $CGRP_1$ and $CGRP_2$ receptors, respectively (Wisskirchen *et al*., 1998). However, also in the rat, the antagonist had an affinity below 5.0 in the aorta consistent with neither a $CGRP_1$ nor a $CGRP_2$ receptor (Wisskirchen *et al*., 1999b). A wide range of affinity values for hα $CGRP_{8-37}$ has been reported from a number of other species, and in some preparations values exceed those for the proposed $CGRP_1$ receptor. For instance, hα $CGRP_{8-37}$ had a pA_2 value of 8.7 in human SK-N-MC cells (Longmore *et al*., 1994), and in the internal anal sphincter (IAS) of the opossum the pA_2 was 8.1 (Chakder & Rattan, 1991). Therefore, some of the reported affinities for hα $CGRP_{8-37}$ may reflect species differences between CGRP receptors.

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To investigate this possibility, the present study was undertaken to examine the pharmacology of CGRP receptors in the isolated IAS of the rat, to compare with values from the opossum. It proved impossible to compare the two species directly, since the necessary opossum tissue could not be obtained, and therefore this study compared the rat smooth muscle with literature data from the opossum IAS. To characterize the CGRP receptor in the rat IAS, the current study investigated the effects of various CGRP agonists, VIP, the antagonist effect of hα $CGRP_{8-37}$ and its analogues, and the effect of peptidase inhibitors on CGRP activity. The present results support a $CGRP_2$ receptor in the rat IAS, similar to that found in the rat vas deferens but differing from the CGRP receptor in the opossum IAS.

Methods

Male Sprague Dawley rats (300–450 g) were stunned and killed by cervical dislocation. The anal canal was isolated, faeces were removed, and the tissue was opened flat with an incision along the longitudinal axis of the canal. The mucosa was removed, while care was taken to avoid disrupting the continuity and integrity of the circular muscle fibres. From the lowermost portion of the anal canal an IAS smooth muscle strip (approximately 2–3 mm width and 0.5–0.7 cm length) was obtained by removing extraneous tissue, connective tissue and the external anal sphincter skeletal muscle by sharp

dissection. The IAS strip was suspended in a tissue bath, under 0.5 g resting tension in Krebs solution containing (mM): Na^+ 143, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128, HCO_3^-25, HPO_4^{2-}1.2, SO_4^{2-}1.2 and glucose 11, at 37°C, gassed with 95% O_2 and 5% CO_2, and equilibrated for at least 60 min. Tension was recorded with Grass FT.03 isometric transducers connected to a Grass 7D polygraph.

During the equilibration time, the smooth muscle began to develop spontaneous tone which plateaued within 30–100 min. Once the plateau was achieved and sustained for at least 15 min, the tissues were washed and spontaneous tone was allowed to recover. A cumulative concentration response curve to one agonist (hα CGRP, hβ CGRP, rat β CGRP, [Cys(ACM^{2,7})] hα CGRP, salmon calcitonin, or VIP) was constructed with successive doses being added when the maximum effect of a given concentration appeared to have been reached. A second curve to each agonist was obtained after washing and recovery of the spontaneous tone (approximately 40 min). The effect of TTX (10^{-6} M; 10 min pretreatment) was examined on spontaneous tone and on second concentration response curves to hα CGRP. In separate experiments, hα CGRP$_{8-37}$ (10^5 M) was equilibrated for 20 min before a second curve to an agonist was constructed. The effects of hβ CGRP$_{8-37}$ and hα CGRP$_{8-37}$ analogues (10^{-5} M; 20 min pretreatment) were studied on second curves to hα CGRP. Hα CGRP$_{8-37}$ analogues were substituted at the N-terminus by either glycine (hα CGRP$_{8-37}$Gly^8), *des*-NH_2 valine (hα CGRP$_{8-37}$ *des*-NH_2 Val^8) or proline (hα CGRP$_{8-37}$ Pro^8). All CGRP analogues were tested on spontaneous tone (up to 10^{-5} M).

A mixture of the peptidase inhibitors amastatin, bestatin, captopril, phosphoramidon and thiorphan (10^{-6} M each; 30 min pretreatment) was studied on responses to either hα CGRP or [Cys(ACM^{2,7})] hα CGRP, and in the presence of hα CGRP$_{8-37}$ to either hα CGRP or rat β CGRP. Responses to hα CGRP and [Cys(ACM^{2,7})] hα CGRP in the absence and presence of peptidase inhibitors were examined within a single tissue. For the antagonist hα CGRP$_{8-37}$ (10^{-5} M) assayed against hα CGRP or rat β CGRP, peptidase inhibitors were present throughout the experiment and compared with results obtained in their absence. The effect of peptidase inhibitors (in DMSO) and DMSO alone was tested on the spontaneous tone.

Chemicals

Amastatin, bestatin, captopril, phosphoramidon, thiorphan, and the peptides [Cys(ACM^{2,7})] hα CGRP and salmon calcitonin were obtained from Sigma, U.K. TTX was obtained from Calbiochem, U.K. Hα CGRP, hβ CGRP, rat β CGRP, hβ CGRP$_{8-37}$, hα CGRP$_{8-37}$ and its analogues were donated by GlaxoWellcome Research Laboratories (Beckenham, Kent, U.K.), having been synthesized and purified as described previously (Wisskirchen *et al*., 1999a). The peptides were dissolved and diluted in distilled water to form a 10^{-2} M stock solution, and stored in aliquots at −20°C. TTX was dissolved and diluted in distilled water. The peptidase inhibitors were dissolved and diluted in DMSO to form a stock solution of 10^{-4} M, and were kept stored at −20°C.

Data analysis

All values are given as mean ± s.e.mean. Responses to relaxant drugs in the IAS, are expressed as a percentage relaxation of the spontaneous tone. Differences were tested for significance using one-way ANOVA and Student's *t*-test for paired and unpaired groups as appropriate. For all tests the significance level was set as $P<0.05$.

The pEC_{50} (−logM of EC_{50}; concentration of the agonist that produced 50% of the maximal relaxation) was determined by non-linear regression curve fitting, using Graphpad Prism 2.0 (Graphpad Software, U.S.A.). The Hill slope of each non-linear regression curve was determined, using Graphpad Prism 2.0, to check whether agonist curves in the absence and presence of antagonists were parallel. Apparent pK_B values for the CGRP antagonists (at a single concentration), were calculated using the following equation:

$$pK_B = -\log [B] + \log (CR - 1)$$

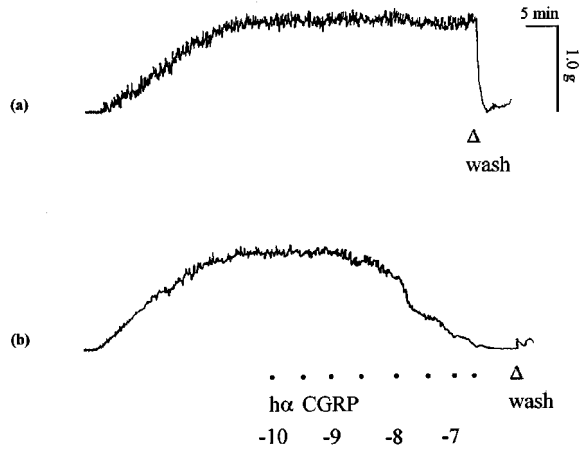

Figure 1 Relaxation to hα CGRP of spontaneous tone in rat internal anal sphincter (IAS). Traces showing (a) development and plateau of spontaneous contractions, and (b) concentration-dependent relaxation to cumulatively administered hα CGRP on spontaneous tone, added in half-log molar increments. Numbers represent log molar concentrations of hα CGRP.

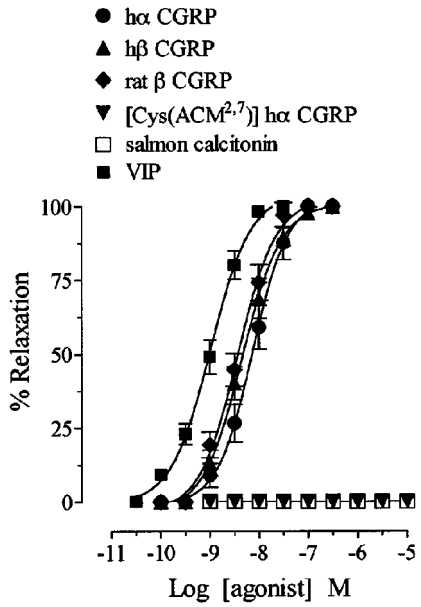

Figure 2 Agonist activities of CGRP analogues, homologues and VIP in rat IAS. Concentration response curves to hα CGRP, hβ CGRP, rat β CGRP, [Cys(ACM^{2,7})] hα CGRP, salmon calcitonin, and vasoactive intestinal polypeptide (VIP) on spontaneous tone. Results are expressed as percentage relaxation of the spontaneous tone. Points and error bars represent the mean ± s.e.mean of four or five separate experiments.

Table 1 Agonist relative potencies of hα CGRP, related peptides and VIP causing relaxation of spontaneous tone in the rat IAS

Agonist	*pEC_{50}*	*E_{max} (%)*	*Hill slope*	*RP (%)*
hα CGRP	8.1±0.2	100	1.2±0.1	100
hβ CGRP	8.3±0.1	100	1.0±0.1	164
rat β CGRP	8.4±0.2	100	1.1±0.1	202
[Cys(ACM^{2,7})]hα CGRP	<5	⩾0	–	<0.02
Salmon calcitonin	<5	⩾0	–	<0.02
VIP	9.0±0.1	100	1.0±0.1	715

PEC_{50} values, the concentrations of peptides required to produce 50% of the maximum effect; E_{max} (%), the maximum effects expressed as percentage relaxation of the spontaneous tone; Hill slope, the slope of the agonist curves; RP (%), relative potency compared with hα CGRP (=100%). Results are obtained from four or five individual tissues where values represent the mean±s.e.mean.

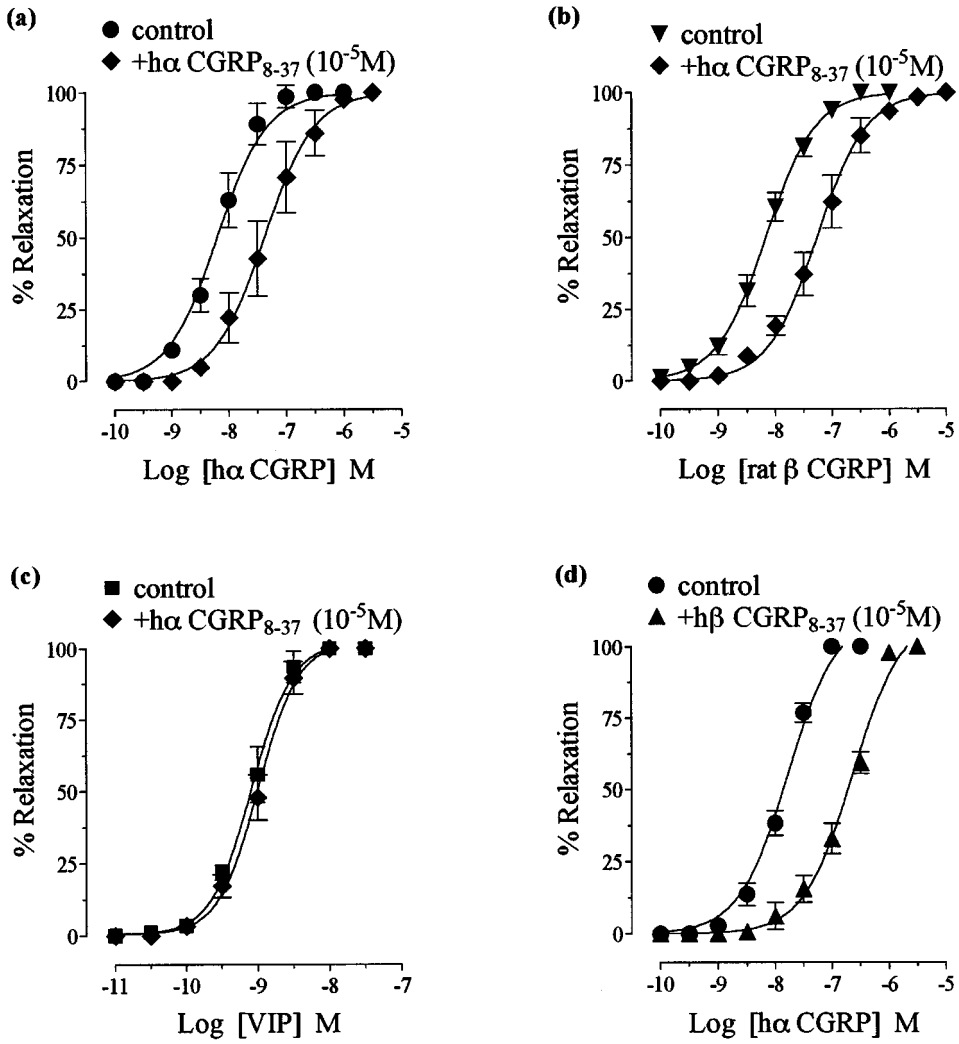

Figure 3 The effect of hα $CGRP_{8-37}$ on hα CGRP, rat β CGRP and VIP responses and the effect of hβ $CGRP_{8-37}$ on hα CGRP responses in the rat IAS. Concentration response curves to (a,d) hα CGRP, (b) rat β CGRP and (c) VIP on spontaneous tone, and in the presence of 10^{-5} M of (a–c) hα $CGRP_{8-37}$ and (d) hβ $CGRP_{8-37}$. Results are expressed as percentage relaxation of the spontaneous tone. Points and error bars represent the mean±s.e.mean of four or five individual experiments.

where [B] is the molar concentration of the antagonist and CR is the concentration ratio of the EC_{50} values in the presence and absence of the antagonist.

Results

In rat isolated IAS strips, a spontaneous contraction developed, which plateaued (Figure 1a) with a tone of 1.2±0.2 g (n=10), and was maintained for at least 40 min.

Agonist activities of CGRP peptides and VIP

Cumulative addition of hα CGRP induced a concentration-dependent relaxation of the spontaneous tone (Figure 1b), with a pEC_{50} value of 8.1±0.2, and a maximum response of 100% relaxation (Figure 2). The effect of a given concentration began within 35–50 s and reached its maximum after about 4–5 min. Agonist responses to hβ CGRP and rat β CGRP were similar to those of hα CGRP (Figure 2; Table 1). The linear

analogue [Cys(ACM^{2,7})] hα CGRP produced no relaxation up to 10^{-5} M (Figure 2), and was at least 5000 fold weaker than hα CGRP (Table 1). Similarly, salmon calcitonin was inactive up to 10^{-5} M (Figure 2a). Vasoactive intestinal polypeptide (VIP) produced relaxation, which was around 7 fold more potent than hα CGRP (Figure 2; Table 1). The onset and equilibration of VIP relaxation was similar to that of hα CGRP.

A second curve to hα CGRP was not significantly different from the control ($P>0.05$; data not shown). Similarly, concentration response curves to hβ CGRP, rat β CGRP or VIP were all reproducible ($P>0.05$; data not shown).

Effect of TTX on IAS tone and on relaxation to hα CGRP

TTX (10^{-6} M; 10 min pretreatment) caused no significant change in the spontaneous tone of the IAS (tension of 1.0 ± 0.2 and 1.1 ± 0.3 g ($n=4$ each) in the absence and presence of TTX, respectively). The relaxant effect of hα CGRP on spontaneous tone was not modified in the presence of the neurotoxin (hα CGRP at 10^{-9} M, 3×10^{-9} M and 10^{-8} M produced 9.1 ± 4.1, 26.7 ± 5.3 and $59.2\pm6.4\%$ relaxation compared with 7.8 ± 2.9, 22.9 ± 4.9 and $60.5\pm7.9\%$ ($n=4$ each), in the absence and presence of TTX, respectively). Similarly, TTX (10^{-6} M) had no significant effect on concentration response curves to hβ CGRP, rat β CGRP or VIP ($P>0.05$; data not shown). (As a control of the effectiveness of TTX neural block, IAS relaxation induced by electrical field stimulation (40 V, 2 ms for 4 s, 0.5–20 Hz; producing $87.1\pm6.6\%$ maximum relaxation; $n=4$) was completely abolished by the neurotoxin, used as above).

Antagonist effect of human CGRP$_{8-37}$ and analogues

Addition of either hα CGRP$_{8-37}$ or one of its analogues (10^{-7}–10^{-5} M) had no effect on spontaneous tone. Pretreatment (20 min) with hα CGRP$_{8-37}$ (10^{-7}–3×10^{-6} M) did not significantly alter concentration response curves to hα CGRP ($P>0.05$). Hα CGRP$_{8-37}$ (10^{-5} M) inhibited hα CGRP responses and produced a parallel rightward shift of the agonist curve without reducing the maximum response (Figure 3a; Table 2). Similarly, hα CGRP$_{8-37}$ (10^{-5} M) was an antagonist against rat β CGRP (Figure 3b; Table 2), while responses to VIP were unaltered by hα CGRP$_{8-37}$ (10^{-5} M; Figure 3c). The β-form of human CGRP$_{8-37}$ (10^{-5} M) antagonized hα CGRP responses, and shifted the agonist curve to the right with no depression in the maximum response

Table 2 Comparison of antagonist affinities for human CGRP$_{8\text{-}37}$ and analogues against CGRP peptides between the rat IAS, the rat vas deferens and the rat pulmonary artery

		pA$_2$/pK$_B$ value*		
Agonist	*Antagonist*	*IAS*	*Vas deferens*	*Pulmonary artery*
hα CGRP	hα CGRP$_{8\text{-}37}$	5.7 ± 0.3*	6.0	6.9
rat β CGRP	hα CGRP$_{8\text{-}37}$	5.8 ± 0.2*	5.8 ± 0.1*	ND
hα CGRP	hβ CGRP$_{8\text{-}37}$	6.1 ± 0.1*	5.6	7.1 ± 0.1*
hα CGRP	hα CGRP$_{8\text{-}37}$ Gly^8	5.8 ± 0.1*	6.1 ± 0.1*	6.9 ± 0.1*
hα CGRP	hα CGRP$_{8\text{-}37}$ *des* NH$_2$ Val^8	5.8 ± 0.1*	6.5 ± 0.1*	7.0 ± 0.1*
hα CGRP	hα CGRP$_{8\text{-}37}$ Pro^8	5.5 ± 0.1*	6.1 ± 0.1*	7.0

Apparent pK_B values (*) were obtained from the concentration ratio using either 10^{-5} M of the antagonists in the IAS and vas deferens, or 10^{-6} M of the antagonists in the pulmonary artery, and are expressed as the mean±s.e.mean. pA$_2$ values were obtained from a Schild plot. Values represent data from four to eight individual experiments. Affinity values for hα CGRP$_{8\text{-}37}$ and analogues from the rat vas deferens and pulmonary artery were obtained from previous reports (Wisskirchen *et al*., 1998; 1999a; 2000); ND, not determined.

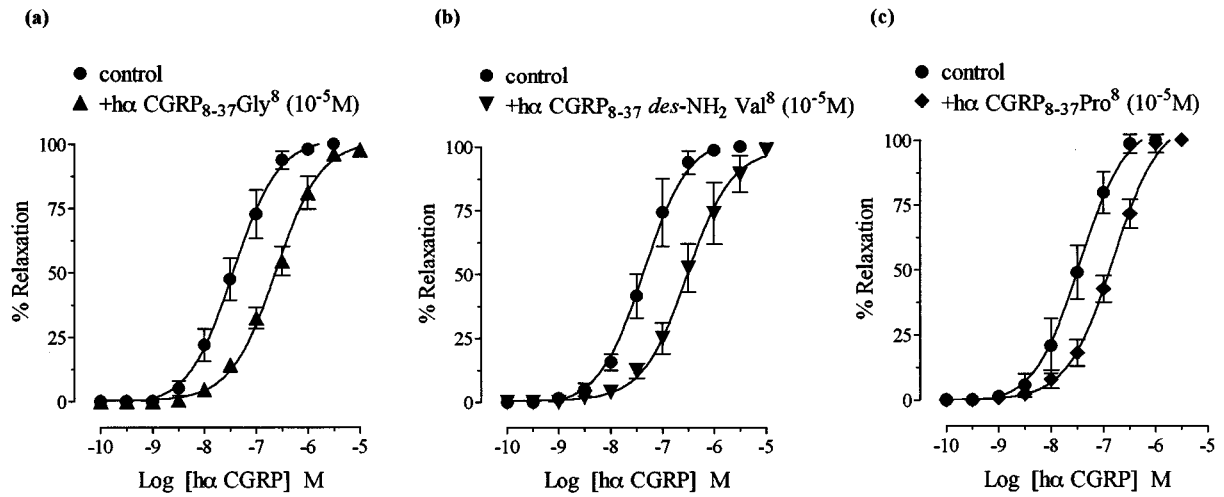

Figure 4 The effect of hα CGRP$_{8-37}$ analogues substituted at the N-terminus on hα CGRP responses in rat IAS. Concentration response curves to hα CGRP on spontaneous tone, and in the presence of 10^{-5} M of (a) hα CGRP$_{8-37}$Gly^8, (b) hα CGRP$_{8-37}$ *des*-NH$_2$ Val^8, and (c) hα CGRP$_{8-37}$Pro^8. Results are expressed as percentage relaxation of the spontaneous tone. Points and error bars represent the mean±s.e.mean of four individual experiments.

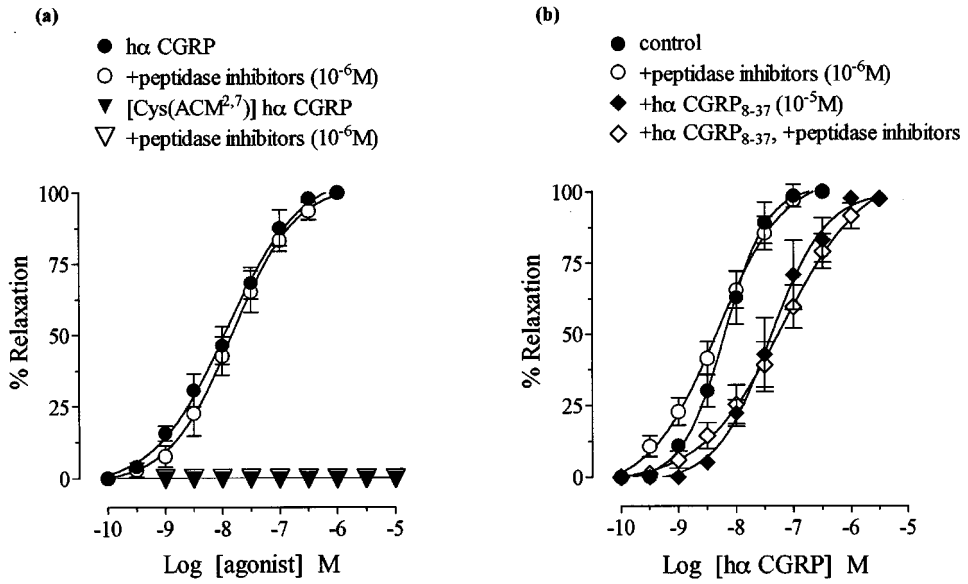

Figure 5 Effect of peptidase inhibitors (amastatin, bestatin, captopril, phosphoramidon, thiorphan; 10^{-6} each) on hα CGRP and [Cys(ACM^{2,7})] hα CGRP responses and on antagonism by hα CGRP$_{8-37}$ against hα CGRP in rat IAS. Concentration response curves to (a) hα CGRP, [Cys(ACM^{2,7})] hα CGRP, and to (b) hα CGRP in the absence and presence of hα CGRP$_{8-37}$ (10^{-5} M) on spontaneous tone, before and after treatment with peptidase inhibitors. Results are expressed as percentage relaxation of the spontaneous tone. Points and error bars represent the mean ± s.e.mean of four individual experiments.

(Figure 3d; Table 2). Hα CGRP$_{8-37}$ analogues (10^{-5} M) with substitutions at the N-terminus by either glycine (hα CGRP$_{8-37}$Gly^8), *des*-NH$_2$ valine (hα CGRP$_{8-37}$*des*-NH$_2$ Val^8) or proline (hα CGRP$_{8-37}$Pro^8) were all antagonists against hα CGRP, with similar apparent pK_B values (Figure 4; Table 2).

Effect of peptidase inhibitors

Addition of either DMSO or a mixture of peptidase inhibitors (amastatin, bestatin, captopril, phosphoramidon, thiorphan; 10^{-6} M each) in DMSO did not alter the spontaneously developed tone of the IAS. The peptidase inhibitors did not modify either the agonist activity of hα CGRP or the inactivity of [Cys(ACM^{2,7})] hα CGRP (Figure 5a). The pEC$_{50}$ values for hα CGRP were 7.9 ± 0.1 and 7.8 ± 0.1 in the absence and presence of peptidase inhibitors, respectively. Similarly, the presence of the peptidase inhibitors did not change the apparent antagonist affinity for hα CGRP$_{8-37}$ against either hα CGRP (Figure 5b) or rat β CGRP. The apparent pK_B values for hα CGRP$_{8-37}$ (10^{-5} M) were 5.7 ± 0.3 and 5.9 ± 0.1 against hα CGRP, and 5.8 ± 0.2 and 6.0 ± 0.1 (n = 4 each) against rat β CGRP, before and after treatment with peptidase inhibitors, respectively.

Discussion

This study supports the presence of CGRP$_2$ receptors in the IAS of the rat, as the apparent affinity for hα CGRP$_{8-37}$ is consistent with that found in rat vas deferens. However, the antagonist potency is 100 fold lower than that reported for the opossum IAS (Chakder & Rattan, 1991).

In the rat IAS, CGRP causes concentration-dependent and TTX-resistant relaxation of the spontaneous tone, consistent with a direct action on the smooth muscle. Hα CGRP$_{8-37}$ antagonizes hα CGRP and not VIP responses, supporting a selective interaction at CGRP receptors. The affinity of hα CGRP$_{8-37}$ in the rat IAS agrees well with reported values from the rat vas deferens (Table 2; Dennis *et al*., 1990; Maggi *et al*., 1991; Mimeault *et al*., 1991; 1992; Wisskirchen *et al*., 1998), but is about 10 fold lower than found previously in the rat pulmonary artery (Table 2; Wisskirchen *et al*., 1998). The similarity between the receptors in the rat IAS and vas deferens is supported by two additional points. Firstly, both human α- and rat β forms of CGRP act on the same class of CGRP receptors in the IAS, since the action of the two peptides was antagonized by hα CGRP$_{8-37}$ in an agonist-independent manner, consistent with a common site of action. Secondly, the β-form of human CGRP$_{8-37}$ appears to recognize the same population of CGRP receptors, since the antagonist had a similar affinity to hα CGRP$_{8-37}$. Thus, in agreement with recent studies in the rat vas deferens (Wisskirchen *et al*., 1998), the present findings in the rat IAS suggest a single class of CGRP$_2$ receptors, that is agonist (hα CGRP, rat β CGRP) and antagonist (hα- and hβ CGRP$_{8-37}$) independent.

For hα CGRP$_{8-37}$, deletion of the N-terminus (valine^8) does not alter antagonism at CGRP receptors in either the guinea-pig atrium or rat vas deferens (Mimeault *et al*., 1991; 1992). Hα CGRP$_{8-37}$ analogues with structural modifications at the N-terminus are equally potent with hα CGRP$_{8-37}$ in rat tissues (Wisskirchen *et al*., 1999a; 2000). To investigate whether this is also true for the CGRP receptor in the rat IAS, the present study examined the activity of these hα CGRP$_{8-37}$ analogues. The data confirm that the substitutions at the N-terminus do not alter the affinity of hα CGRP$_{8-37}$ suggesting that valine^8 neither interacts with receptor binding sites nor plays a structural role for antagonism by hα CGRP$_{8-37}$. The similarity between the affinity values for the hα CGRP$_{8-37}$ analogues in this study and those previously reported in the rat vas deferens (Wisskirchen *et al*., 1999a; Table 2) supports the presence of CGRP$_2$ receptors in the rat IAS.

In several other species/tissues, CGRP$_2$ receptors have also been suggested, including the mouse aorta (Quirion *et al*., 1992), guinea-pig bladder (Giuliani *et al*., 1992), guinea-pig vas deferens (Tomlinson & Poyner, 1996) and human COL 27 cells (Cox & Tough, 1994). However, these studies did not determine an affinity for hα CGRP$_{8-37}$ but only showed its relative inactivity (using no higher concentrations than

3×10^{-6} M or 10^{-6} M). This is an important point as an even higher concentration of hα CGRP$_{8-37}$ (10^{-5} M) failed to antagonize CGRP-evoked relaxation of the rat aorta (Wisskirchen *et al*., 1999b). Therefore it may be misleading to equate the relative lack of activity of the antagonist as evidence for the presence of a CGRP$_2$ receptor. In these circumstances the routine use of higher concentrations of hα CGRP$_{8-37}$ would either provide support for the existence of a CGRP$_2$ receptor or make this possibility unlikely as seen in the aorta.

In the opossum IAS, hα CGRP$_{8-37}$ selectively antagonized CGRP responses (pA$_2$ values of 7.8 and 8.1 against hα CGRP and hβ CGRP, respectively; Chakder & Rattan, 1991). This antagonist affinity, however, is around 10 fold higher than that at the CGRP$_1$ receptor in the guinea-pig right atrium (pA$_2$ 7.2 against hα CGRP; Mimeault *et al*., 1991), and up to 100 fold higher than at the CGRP$_2$ receptor in the rat vas deferens. The present study in the rat IAS demonstrates an apparent affinity value for hα CGRP$_{8-37}$ which is also at least 100 fold lower than in the same tissue in the opossum. Therefore, these results may highlight species differences which could partly explain the range of hα CGRP$_{8-37}$ affinities. Since there is no literature data on other opossum tissues, the affinity for hα CGRP$_{8-37}$ reported in the opossum IAS could represent either a CGRP$_1$ or a CGRP$_2$ receptor or alternatively, yet another CGRP receptor subtype.

In the opossum IAS there was tachyphylaxis to CGRP (Chakder & Rattan, 1991) and this was not observed in the rat tissue, suggesting either different receptors and/or different receptor effector mechanisms. While VIP is (approximately 1000 fold) more potent in the rat IAS (pEC$_{50}$ 9.0) than in the opossum tissue (estimated pEC$_{50}$ 6.2; Chakder & Rattan, 1991), the potency of the CGRP peptides in the rat IAS (pEC$_{50}$ 8.1–8.4) and the opossum IAS (pEC$_{50}$ 7.5–8.5; Chakder & Rattan, 1990; 1991) were similar.

The linear analogue [Cys(ACM^{2,7})] hα CGRP has been suggested to be a selective agonist at the CGRP$_2$ receptor (Dennis *et al*., 1989), although this was not confirmed by previous studies in the rat vas deferens (Wisskirchen *et al*., 1998). In the rat IAS, the native CGRP peptides (hα-, hβ- and rat β CGRP) are full agonists, while [Cys(ACM^{2,7})] hα CGRP was inactive (up to 10^{-5} M). In the presence of several peptidase inhibitors, [Cys(ACM^{2,7})] hα CGRP was no more effective, suggesting it was not rapidly broken down. Therefore, the present results suggest that [Cys(ACM^{2,7})] hα CGRP is not a helpful tool unlike hα CGRP$_{8-37}$ and its analogues with which to characterize CGRP$_2$ receptors. A similar conclusion about [Cys(ACM^{2,7})] hα CGRP and hα CGRP$_{8-37}$ analogues was previously reached for the CGRP$_2$ receptor in the vas deferens (Wisskirchen *et al*., 1998; 1999a).

Peptidase inhibitors, such as thiorphan and phosphoramidon increased the effect of CGRP agonists or antagonists in some tissues (Longmore *et al*., 1994; Maggi & Giuliani, 1994; Chatelain *et al*., 1995). Thus, a differing enzyme distribution might explain the difference in hα CGRP$_{8-37}$ affinity between the present study and that in the opossum. However, it appears that peptidase inhibitors do not alter the affinity for hα CGRP$_{8-37}$ in the rat IAS, since neither the potency of hα CGRP nor the antagonist activity of hα CGRP$_{8-37}$ was increased. Similar to the present findings but in contrast to those by Longmore *et al*. (1994), reported affinity values for hα CGRP$_{8-37}$ in the rat vas deferens are not significantly different in the presence (Giuliani *et al*., 1992; Wisskirchen *et al*., 1998) or absence of peptidase inhibitors (Wisskirchen *et al*., 1998; Maggi *et al*., 1991; Mimeault *et al*., 1991; 1992). Therefore, in agreement with these literature data, the affinity for hα CGRP$_{8-37}$ in this study appears not to be low due to peptidergic degradation, but supports the presence of a CGRP$_2$ receptor subtype in the rat IAS.

Another possible explanation for the difference in hα CGRP$_{8-37}$ affinity between the rat and the opossum IAS could be that the fragment did not reach equilibrium at its receptor (after 20 min) in the present experiments. However, in other rat smooth muscles such as the pulmonary artery and the vas deferens it appears that hα CGRP$_{8-37}$ reaches equilibrium within 2–3 min (Wisskirchen *et al*., 1998).

In this study, apparent pK_B values have been determined from one measurement of concentration ratios, and so must be interpreted with caution since competitive antagonism has been assumed. However, despite the limitation, all apparent pK_B values for hα CGRP$_{8-37}$ and analogues are in good agreement with pA$_2$ values for CGRP$_{8-37}$ (hα and β) in the vas deferens (Table 2).

The protein CRLR (calcitonin receptor-like receptor) when co-expressed with a receptor activity modifying protein (RAMP1) has a CGRP$_1$-like pharmacology rather than that of a CGRP$_2$ receptor (Aiyar *et al*., 1996; McClatchie *et al*., 1998). However the latter receptor could reflect differences in either CRLR or RAMP or both. The combination of CRLR with either RAMP2 or RAMP3 produced an adrenomedullin-like pharmacology (McLatchie *et al*., 1998; Fraser *et al*., 1999). However it remains possible that the range of CGRP$_{8-37}$ affinities might be due to a combination of CRLR with different proportions of RAMP1, 2 and 3 in different systems. If either CRLR or RAMPs are involved in mediating the relaxation to CGRP in the IAS, differences in their structures between species may explain the present results.

It has recently been reported that the novel CGRP receptor antagonist BIBN4096BS has over 200 fold selectivity in favour of primate (human SK-N-MC cells) over rodent (rat spleen) CGRP receptors (Doods *et al*., 2000). While these findings are consistent with a species difference between the receptors it remains possible that there might be another explanation for the 'selectivity', for example CGRP receptor subtypes.

In conclusion, part of the variability in hα CGRP$_{8-37}$ affinities reported from the literature may be due to species differences, as seen in the IAS between rat and opossum. Therefore, caution has to be taken in classifying CGRP receptors when comparing across different species.

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