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Pharmacological characterization of CGRP receptors mediating relaxation of the rat pulmonary artery and inhibition of twitch responses of the rat vas deferens

F.M. Wisskirchen, R.P. Burt & [1]I. Marshall

Department of Pharmacology, University College London, Gower Street, London WC1E 6BT

1 CGRP receptors mediating vasorelaxation of the rat isolated pulmonary artery and inhibition of contractions of the rat isolated prostatic vas deferens were investigated using CGRP agonists, homologues and the antagonist $CGRP_{8-37}$.

2 In the pulmonary artery, human (h)α-CGRP-induced relaxation of phenylephrine-evoked tone was abolished either by removal of the endothelium or by N^G-nitro-L-arginine (10^{-5} M). The inhibitory effect of N^G-nitro-L-arginine was stereoselectively reversed by L- but not by D-arginine (10^{-4} M). Thus, CGRP acts via nitric oxide released from the endothelium.

3 In the endothelium-intact artery, hα-CGRP, hβ-CGRP and human adrenomedullin (10^{-10}–3×10^{-7} M), dose-dependently relaxed the phenylephrine-induced tone with similar potency. Compared with hα-CGRP, rat amylin was around 50 fold less potent, while [Cys(ACM^{2,7})] hα-CGRP (10^{-7}–10^{-4} M) was at least 3000 fold less potent. Salmon calcitonin was inactive (up to 10^{-4} M).

4 Human α-$CGRP_{8-37}$ (3×10^{-7}–3×10^{-6} M) antagonized hα-CGRP (pA_2 6.9, Schild plot slope 1.2±0.1) and hβ-CGRP (apparent pK_B of 7.1±0.1 for hα-$CGRP_{8-37}$ 10^{-6} M) in the pulmonary artery. Human β-$CGRP_{8-37}$ (10^{-6} M) antagonized hα-CGRP responses with a similar affinity (apparent pK_B 7.1±0.1). Human adrenomedullin responses were not inhibited by hα-$CGRP_{8-37}$ (10^{-6} M).

5 In the prostatic vas deferens, hα-CGRP, hβ-CGRP and rat β-CGRP (10^{-10}–3×10^{-7} M) concentration-dependently inhibited twitch responses with about equal potency, while rat amylin (10^{-8}–10^{-5} M) was around 10 fold less potent and the linear analogue [Cys(ACM^{2,7})] hα-CGRP was at least 3000 fold weaker. Salmon calcitonin was inactive (up to 10^{-4} M).

6 The antagonist effect of hα-$CGRP_{8-37}$ (10^{-5}–3×10^{-5}) in the vas deferens was independent of the agonist, with pA_2 values against hα-CGRP of 6.0 (slope 0.9±0.1), against hβ-CGRP of 5.8 (slope 1.1±0.1), and an apparent pK_B value of 5.8±0.1 against both rat β-CGRP and rat amylin. Human β-$CGRP_{8-37}$ (3×10^{-5}–10^{-4} M) competitively antagonized hα-CGRP responses (pA_2 5.6, slope 1.1±0.2). The inhibitory effect of hα-CGRP on noradrenaline-induced contractions in both the prostatic and epididymal vas deferens was antagonized by hα-$CGRP_{8-37}$ (pA_2 5.8 and 5.8, slope 1.0±0.2 and 1.0±0.3, respectively).

7 The effects of hα-CGRP and hα-$CGRP_{8-37}$ in both rat pulmonary artery and vas deferens were not significantly altered by pretreatment with peptidase inhibitors (amastatin, bestatin, captopril, phosphoramidon and thiorphan, all at 10^{-6} M). The weak agonist activity of [Cys(ACM^{2,7})] hα-CGRP in the vas deferens was not increased by peptidase inhibitors.

8 These data demonstrate that two different CGRP receptors may exist in the rat pulmonary artery and vas deferens, a $CGRP_1$ receptor subtype in the rat pulmonary artery ($CGRP_{8-37}$ pA_2 6.9), while the lower affinity for $CGRP_{8-37}$ (pA_2 6.0) in the vas deferens is consistent with a $CGRP_2$ receptor.

Keywords: $CGRP_1$ receptor; $CGRP_2$ receptor; hα-CGRP; rat β-CGRP; rat amylin; [Cys(ACM^{2,7})] hα-CGRP; human adrenomedullin; hα-$CGRP_{8-37}$; hβ-$CGRP_{8-37}$; peptidase inhibitors

Introduction

Calcitonin gene-related peptide (CGRP) is a 37 residue neuropeptide with numerous biological actions, including dilatation of blood vessels (Brain *et al*., 1985; Holman *et al*., 1986; Marshall *et al*., 1986a,b). As CGRP could play a role in several disorders, there has been interest in characterizing its receptors.

CGRP may act on at least two receptor subtypes, $CGRP_1$ and $CGRP_2$, present in the guinea-pig atrium and rat vas deferens, respectively (Dennis *et al*., 1989; 1990; Mimeault *et al*., 1991; Quirion *et al*., 1992). This classification is based on both agonist relative potency ([Cys(ACM^{2,7})]human (h)α-CGRP may be a weak agonist in the rat vas deferens while being inactive in the guinea-pig atrium; Dennis *et al*., 1989) and antagonist affinity (hα-$CGRP_{8-37}$ is more potent in the guinea-pig atrium, pA_2 7.2–7.7 than in the rat vas, pA_2 6.2; Mimeault *et al*., 1991).

Although this classification has met with some success in rationalizing apparent CGRP receptor heterogeneity, several problems have become apparent: the prototypic $CGRP_1$ and $CGRP_2$ receptors are found in the guinea-pig and rat, respectively, although the extent of species differences is largely undocumented. In some preparations $CGRP_{8-37}$ give pA_2 values between 7.8 and 9.3 (Chakder & Rattan 1991; Poyner *et al*., 1992; Bell & McDermott, 1994; Longmore *et al*., 1994), which exceed those values for the proposed $CGRP_1$ receptor. Furthermore, differing affinities of CGRP analogues may be due to differences in proteolytic enzyme distribution rather than reflecting CGRP receptor subtypes (Longmore *et al*., 1994).

[1] Author for correspondence.

The lack of sufficiently selective CGRP agonists and antagonists has become more of a problem with the discovery of peptides with structural homology to CGRP, such as amylin (Cooper *et al*., 1987) and adenomedullin (Kitamura *et al*., 1993). These peptides can stimulate CGRP receptors (Giuliani *et al*., 1992; Zimmermann *et al*., 1995), although they have their own receptors (Beaumont *et al*., 1993; 1995; Kapas *et al*., 1995).

In order to obviate any species differences the present experiments have used only rat tissues in a systematic investigation of the pharmacology of CGRP receptors, with CGRP agonists and antagonists. The results support $CGRP_1$ and $CGRP_2$ subtypes in the rat pulmonary artery and the vas deferens, respectively. A preliminary account of some of these data has been published (Wisskirchen & Marshall, 1997).

Methods

Male Sprague Dawley rats (300–450 g) were stunned and killed by cervical dislocation. The pulmonary arteries and vasa deferentia were isolated and cleared of fat and connective tissue.

Pulmonary artery

Arteries were cut into rings of approximately 2–3 mm in length. Care was taken to minimize damage to the endothelium whilst suspending the rings on tungsten wires (0.125 mm diameter) under 0.5 g resting tension in organ baths. These were filled with Krebs solution containing (mM): Na^+ 143, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128, HCO_3^- 25, HPO_4^- 1.2, SO_4^{2-} 1.2 and glucose 11, at 37°C, and were gassed with 95% O_2 and 5% CO_2. The rings were allowed to equilibrate for 100 min. Tension was recorded with Grass FT.03 isometric transducers connected to a Grass 7D polygraph.

Phenylephrine (3×10^{-8} M) evoked a submaximal response (50–60% of maximal contraction) the stability of which was assessed over 10 min. Acetylcholine (10^{-6} M) was added and tissues showing less than 80% relaxation were discarded as having partially damaged endothelium.

In some experiments, the endothelium was removed by gently abrading the intimal surface with fine wires. The failure of acetylcholine (10^{-6} M) to elicit a relaxant response (<5% relaxation) of tone induced by phenylephrine (3×10^{-8} M) was taken as an indication of endothelium removal, while subsequent addition of sodium nitroprusside (10^{-6} M) causing 100% relaxation was an indication of an intact functional smooth muscle.

Endothelium-intact rings were subsequently contracted with phenylephrine 100 min later and a cumulative concentration-response curve to one agonist (hα-CGRP, hβ-CGRP, rat β-CGRP, [Cys(ACM^{2,7})] hα-CGRP, rat amylin, human adrenomedullin, salmon calcitonin) was constructed and repeated after another 100 min. N^G-nitro-L-arginine, L-arginine and D-arginine were added 30 min before a second concentration-effect curve to hα-CGRP. In separate experiments, the CGRP antagonists (hα- or hβ-$CGRP_{8-37}$) were equilibrated for 20 min before a repeat curve to an agonist. The effect of equilibrating hα-$CGRP_{8-37}$ (10^{-5} M) for either 2 or 60 min was assessed against hα-CGRP. Human α-$CGRP_{8-37}$ (10^{-7}–10^{-5} M) was tested on the basal tone (i.e. unstimulated preparation) and on the spasmogen-induced tone.

Vas deferens

After bisection, the prostatic half was suspended under 0.5 g resting tension and equilibrated for 75 min in Krebs solution (composition: see above), at 37°C, gassed with 95% O_2 and 5% CO_2. Contractile responses of the prostatic vas were induced by electrical field stimulation at 0.2 Hz, 1.0 ms and 60 V (Grass S48 stimulator) through parallel platinum wire electrodes either side of the tissue. The isometric tone was recorded with a Grass FT.03 transducer as above.

For measurements of agonist relative potency, only one agonist was used per tissue. Contractile responses to field stimulation were tested for stability for 10 min, and 40 min later cumulative dose-response curves to hα-CGRP or to one of its analogues or homologues (hβ-CGRP, rat β-CGRP, [Cys(ACM^{2,7})] hα-CGRP, rat amylin, salmon calcitonin) were constructed. For measurements of antagonist affinity, first curves to agonists were obtained as above and after pretreatment with antagonist a repeat agonist curve was constructed. Between curves, a 40 min recovery time was allowed and before construction of a second agonist concentration-response curve the antagonist hα-$CGRP_{8-37}$ (10^{-5}–3×10^{-5} M) was added to equilibrate with the tissue for 20 min. Only one concentration of antagonist was used in a given tissue. In separate experiments, hα-$CGRP_{8-37}$ (10^{-5} M) was also equilibrated for 3 and 60 min before a second curve to hα-CGRP. Human β-$CGRP_{8-37}$ (3×10^{-5}–10^{-4} M) was studied on hα-CGRP responses after 20 min equilibration. The CGRP fragments were tested on basal tone, i.e. on the unstimulated preparation and on twitch responses.

In some experiments, either the prostatic or epididymal end of the vas deferens was set up as above and contracted with noradrenaline (10^{-5} M, about 70% of the maximal response). After establishing consistent contractions to noradrenaline, hα-CGRP was given 30 s before the spasmogen to inhibit the contractile response. Human α-CGRP was given in increasing concentrations in a non-cumulative manner because the contraction to noradrenaline was phasic. There was 10 min between successive applications of noradrenaline. After the control inhibition curve to hα-CGRP either the curve was repeated one hour later or after 40 min hα-$CGRP_{8-37}$ was added to the Krebs solution and after a further 20 min the hα-CGRP curve was repeated.

Peptidase inhibitors

In both smooth muscle preparations, 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 alone or to hα-CGRP in the presence of hα-$CGRP_{8-37}$. In the vas, the effect of the peptidase inhibitors was also examined on [Cys(ACM^{2,7})] hα-CGRP. For the agonists, responses in the absence and presence of peptidase inhibitors were examined successively within a single tissue, while for the antagonist hα-$CGRP_{8-37}$ (10^{-5} M) assayed against hα-CGRP, peptidase inhibitors were present throughout the experiment and compared with results obtained in their absence.

Chemicals

Amastatin, bestatin, captopril, phosphoramidon, thiorphan, rat amylin, [Cys(ACM^{2,7})] hα-CGRP, human adrenomedullin, salmon calcitonin, noradrenaline bitartrate and phenylephrine hydrochloride were obtained from Sigma (U.K.). Human

α-CGRP, hβ-CGRP, rat β-CGRP, hα-$CGRP_{8-37}$ and hβ-$CGRP_{8-37}$ were donated by Glaxo-Wellcome Research Laboratories (Beckenham, Kent), having been synthesized on an ABI 430 peptide synthesizer utilizing FastMoc chemistry, cleaved and de-protected by conventional protocols, purified to homogeneity by reverse phase-high performance liquid chromatography (r.p.-h.p.l.c.) and fully characterized by high field nuclear magnetic resonance (n.m.r.) and mass spectrometry. All peptides were diluted in distilled water to form a 10^{-2} M stock solution and stored in aliquots at −20°C. The peptidase inhibitors were diluted in dimethylsulphoxide (DMSO), to form a stock solution of 10^{-4} M and kept stored at −20°C. Noradrenaline (with added ascorbic acid to prevent oxidation) and phenylephrine were prepared daily in distilled water (10^{-3} M). N^G-nitro-L-arginine was dissolved in HCl (1 M), pH adjusted to 7.4, and diluted with distilled water to form a stock solution (10^{-3} M).

Data analysis

All values are given as mean ± s.e.mean. Responses to vasodilators in the pulmonary artery are expressed as a percentage relaxation of the spasmogen-induced tone. The reduction in twitch tension of the field-stimulated prostatic vas deferens in response to applied drugs is expressed as a percentage of the twitch responses before drug addition. Inhibition of the noradrenaline-induced contractions of the vas deferens by the applied drugs is expressed as percentage inhibition of the spasmogen-induced tone. Statistical analysis was by one-way ANOVA followed by Dunnett's test (multiple comparisons) or by Student's *t* test (for paired or unpaired groups) as appropriate, accepting significance at $P<0.05$.

The EC_{50} or IC_{50} (molar concentration of the agonist that produced 50% of the maximal response) was calculated by non-linear regression curve fitting, with Graphpad Prism 2.0 (Graphpad Software, U.S.A.) and these values were used to determine pEC_{50} or pIC_{50} values ($-\log EC_{50}$ or $-\log IC_{50}$). The Hill slope of each non-linear regression curve was determined by use of Graphpad Prism 2.0.

In the presence of an antagonist with a single concentration used, an apparent pK_B value was calculated given by the equation:

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

where [B] is the molar concentration of the antagonist and CR is the concentration ratio of the EC_{50} or IC_{50} values in the presence and absence of the antagonist. Where multiple concentrations of antagonist were used, a Schild plot of log (CR-1) against log [B] was plotted, and the pA_2 and Schild slope determined by linear regression (Graphpad Prism 2.0). The pA_2 values were calculated from the individual control dose-response curves and the respective curves obtained in the presence of (hα- and hβ-) $CGRP_{8-37}$.

Results

In the rat isolated pulmonary artery, phenylephrine (3×10^{-8} M) evoked a stable contraction (Figure 1) of 0.18 ± 0.02 g ($n=24$) and 0.39 ± 0.04 g ($n=9$) in endothelium-intact and -denuded rings, respectively.

In the rat isolated prostatic vas deferens, twitch responses evoked by electrical field stimulation resulted in reproducible uniform phasic contractions with a tension of 1.0 ± 0.1 g ($n=43$). The maximum phasic noradrenaline (10^{-5} M)-induced contraction of the prostatic and epididymal portions of the vas deferens were 0.40 ± 0.03 g and 1.68 ± 0.08 g, respectively (each $n=4$).

Endothelium-dependence of CGRP relaxation in the pulmonary artery

Cumulative addition of hα-CGRP ($10^{-10}-3\times10^{-7}$ M) to the preconstricted artery induced a dose-dependent vasodilator effect only in endothelium-intact rings but not after endothelium removal in 4 experiments (Figure 1). In endothelium-denuded rings, higher concentrations of hα-CGRP (up to 10^{-5} M) did not relax the phenylephrine-induced tone. Pretreatment with the nitric oxide synthase inhibitor N^G-nitro-L-arginine (10^{-5} M; 30 min) in endothelium-intact rings abolished hα-CGRP responses (Figure 2). The inhibitory effect of N^G-nitro-L-arginine was stereoselectively reversed by L-arginine (10^{-4} M), a substrate of nitric oxide synthase, but not by its D-isomer (D-arginine). All subsequent experiments with CGRP were performed in rings with intact endothelium.

Agonist activity of hα-CGRP and related peptides

In the pulmonary artery, hα-CGRP dose-dependently relaxed the phenylephrine-induced tone with a pEC_{50} of 8.5 ± 0.1 and 100% maximum relaxation (Figure 3a; Table 1). The effect of a given concentration began within 5–10 s of administration and reached its maximum after 40–60 s. Agonist responses to hβ-CGRP were similar, and reached the same maximum effect (Table 1). Responses to human adrenomedullin were slower both in onset and to reach a maximum (20 s and around 200 s, respectively), albeit showing a similar potency to CGRP and the same maximum response (Figure 3a; Table 1). Rat amylin was about 50 times less potent than hα-CGRP, while the linear

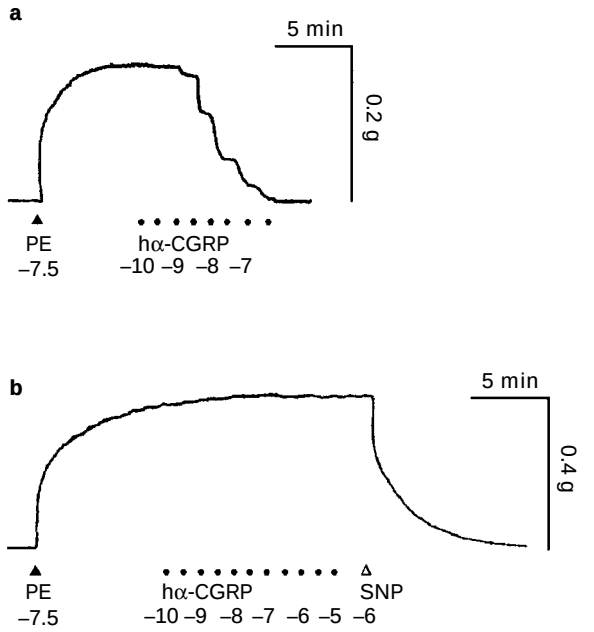

Figure 1 Endothelium-dependent vasorelaxation of hα-CGRP responses in rat isolated pulmonary artery. (a) Trace showing dose-dependent vasorelaxation to cumulatively administered hα-CGRP on phenylephrine-induced tone (PE; 3×10^{-8} M) in an endothelium-intact ring. (b) Trace showing no response to hα-CGRP in an endothelium-denuded phenylephrine-preconstricted ring, while addition of sodium nitroprusside (SNP; 10^{-6} M) relaxes the smooth muscle. Numbers represent log molar concentrations, and hα-CGRP was added in half-log molar increments.

analogue [Cys(ACM^{2,7})] hα-CGRP was at least 3000 fold weaker than hα-CGRP (reaching only 51.6±7.0% relaxation at 10^{-4} M; Figure 3a; Table 1). Salmon calcitonin was inactive up to 10^{-4} M (Figure 3a).

Reproducible relaxation curves were obtained for hα-CGRP, human adrenomedullin or [Cys(ACM^{2,7})] hα-CGRP ($P<0.05$), suggesting little loss of endothelium or receptor desensitization (data not shown).

In the prostatic vas deferens, hα-CGRP caused a concentration-dependent inhibition of twitch responses with a pIC_{50} of 7.9±0.1 and a maximum response of 79.7±3.1% inhibition (Figure 3b). The onset and equilibration of the effect of the peptide occurred after 20–30 s and 90–120 s, respectively. Dose-response curves to the β-forms of human and rat CGRP were similar in time, potency and maximum response as compared with hα-CGRP (Table 1). Rat amylin was less potent than hα-CGRP but produced a similar maximum response (Figure 3b). The linear analogue [Cys(ACM^{2,7})] hα-CGRP showed some agonist activity at high concentrations (Figure 3b) being at least 3000 fold weaker than hα-CGRP (Table 1). Salmon calcitonin had no effect on twitch responses up to 10^{-4} M (Figure 3b).

Non-cumulative addition of hα-CGRP before noradrenaline-induced contractions (10^{-5} M) in the prostatic and epididymal ends of the vas gave pIC_{50} values of 8.5±0.1 and 7.8±0.1 ($n=4$ each), respectively, and curves were reproducible ($P>0.05$; data not shown). Repeat curves to hα-CGRP, rat amylin and [Cys(ACM^{2,7})] hα-CGRP on twitch responses showed no tachyphylaxis ($P>0.05$; data not shown).

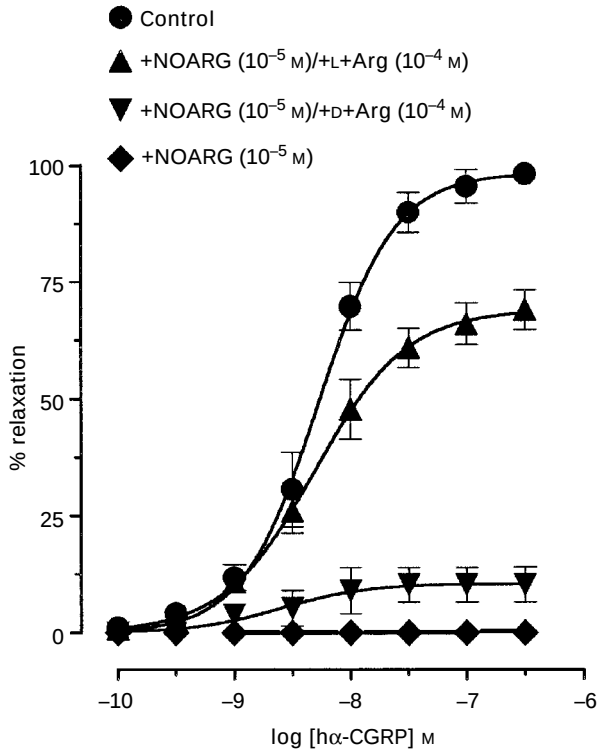

Figure 2 Nitric oxide-dependent pathway of hα-CGRP relaxation in rat endothelium-intact pulmonary artery. Dose-response curve to hα-CGRP alone (control) on the phenylephrine-induced tone, and after pretreatment with N^G-nitro-L-arginine (NOARG; 10^{-5} M). The inhibitory effect of N^G-nitro-L-arginine was partially reversed by L-arginine (L-Arg; 10^{-4} M), but not by D-arginine (D-Arg; 10^{-4} M). Results are expressed as percentage relaxation of the spasmogen-induced tone. Points represent the mean and vertical lines show s.e.mean of 4 experiments.

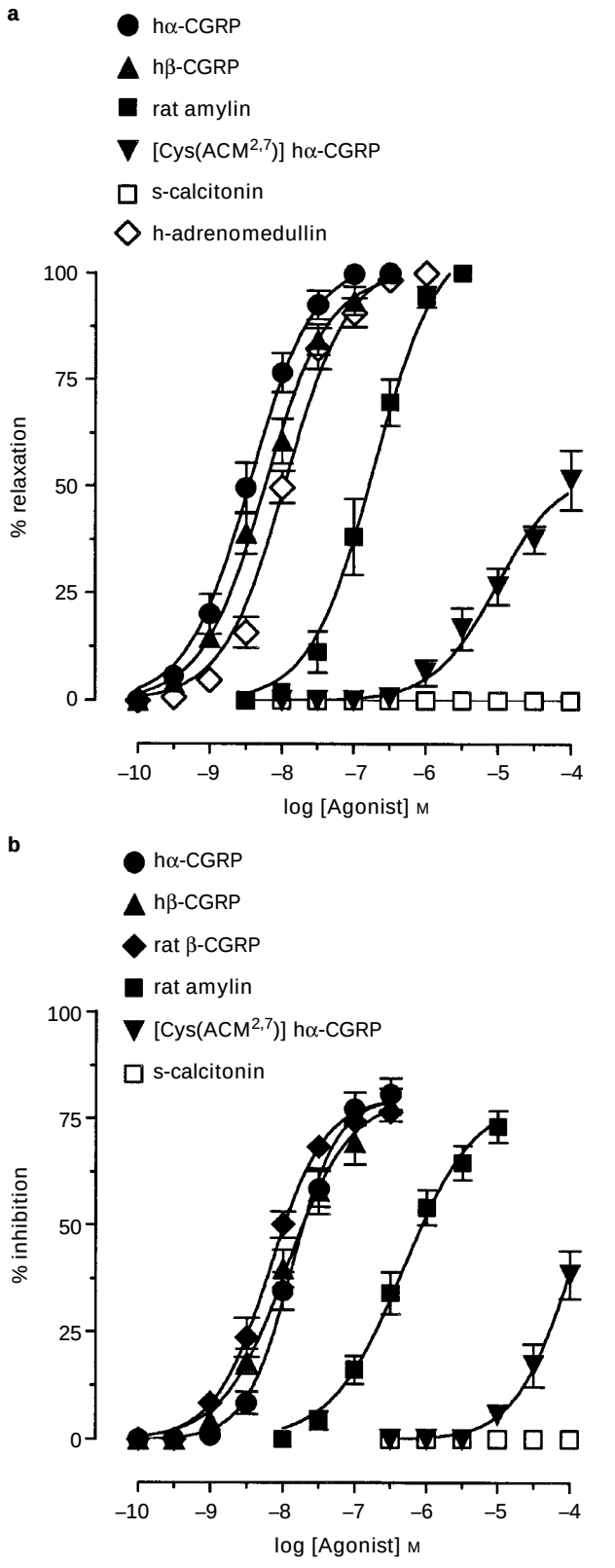

Figure 3 Agonist activities of CGRP analogues and homologues in rat pulmonary artery and vas deferens. Dose-response curves for hα-CGRP, hβ-CGRP, rat β-CGRP (vas deferens, only), human adrenomedullin (h-adrenomedullin pulmonary artery, only), rat amylin, [Cys(ACM^{2,7})] hα-CGRP and salmon calcitonin (s-calcitonin) on (a) phenylephrine-induced tone in the pulmonary artery and on (b) twitch responses in the prostatic vas deferens. Results are expressed as percentage relaxation of the spasmogen-induced tone for the artery and as percentage inhibition of twitch responses for the vas. Points represent the mean and vertical lines show s.e.mean of 4 or 5 experiments.

Antagonism by CGRP$_{8-37}$ in the pulmonary artery

Addition of hα-CGRP$_{8-37}$ (10^{-7}–10^{-6} M) alone had no effect on basal or spasmogen-induced tone in the pulmonary artery, while higher concentrations of the fragment (e.g. 10^{-5} M) caused relaxation of the spasmogen-induced tone (20–50% relaxation; onset and equilibrium varying between 3 and 20 min). Human α-CGRP$_{8-37}$ (10^{-6} M) incubated for either 2, 20 or 60 min, before addition of hα-CGRP (apparent pK_B 7.1±0.1, 7.0±0.1, 6.6±0.2, respectively, $n=4$ each) indicated that equilibrium was reached after 2 min. Thereafter, CGRP fragments were incubated for 20 min before the addition of agonists.

Pretreatment with hα-CGRP$_{8-37}$ (3×10^{-7}–10^{-5} M) inhibited hα-CGRP responses, but did not produce a dose-

Table 1 Agonist relative potencies of hα-CGRP analogues and homologues on vasorelaxation in rat preconstricted pulmonary artery and on inhibition of twitch responses in rat prostatic vas deferens

	Pulmonary artery				*Vas deferens*			
Agonist	*pEC$_{50}$*	*E$_{max}$* (%)	*Hill slope*	*RP* (%)	*pIC$_{50}$*	*I$_{max}$* (%)	*Hill slope*	*RP* (%)
hα-CGRP	8.5±0.1	100±0.0	1.1±0.1	100	7.9±0.1	79.7±3.1	1.2±0.1	100
hβ-CGRP	8.2±0.1	100±0.0	0.9±0.1	57	7.9±0.1	78.2±3.8	1.0±0.1	131
Rat β-CGRP	ND	ND	ND	ND	8.2±0.1	76.5±2.2	1.1±0.1	233
Rat amylin	6.8±0.2	100±0.0	1.2±0.1	2	6.4±0.1	73.2±3.4	1.0±0.1	4
[Cys(ACM^{2,7})] hα-CGRP	<4.8	>51.6±7.0	0.8±0.1	<0.02	<5.2	⩾38.5±5.6	1.2±0.2	<0.03
Salmon calcitonin	<4.0	⩾0.0	ND	<0.003	<4.0	⩾0.0	ND	<0.01
Human adrenomedullin	8.0±0.1	100±0.0	1.2±0.1	33	ND	ND	ND	ND

pEC$_{50}$ or pIC$_{50}$ values, the concentrations of peptides required to induce 50% of the maximum effect; E$_{max}$ or I$_{max}$ (%), the maximum effects expressed as % relaxation of the spasmogen-induced tone (pulmonary artery) or as % inhibition of twitch responses (vas deferens), respectively; Hill slope, the slope of the agonist dose-response curves. Values are mean±s.e.mean from 4 or 5 individual tissues; RP (%), relative potency compared with hα-CGRP (taken as 100%); ND; not determined.

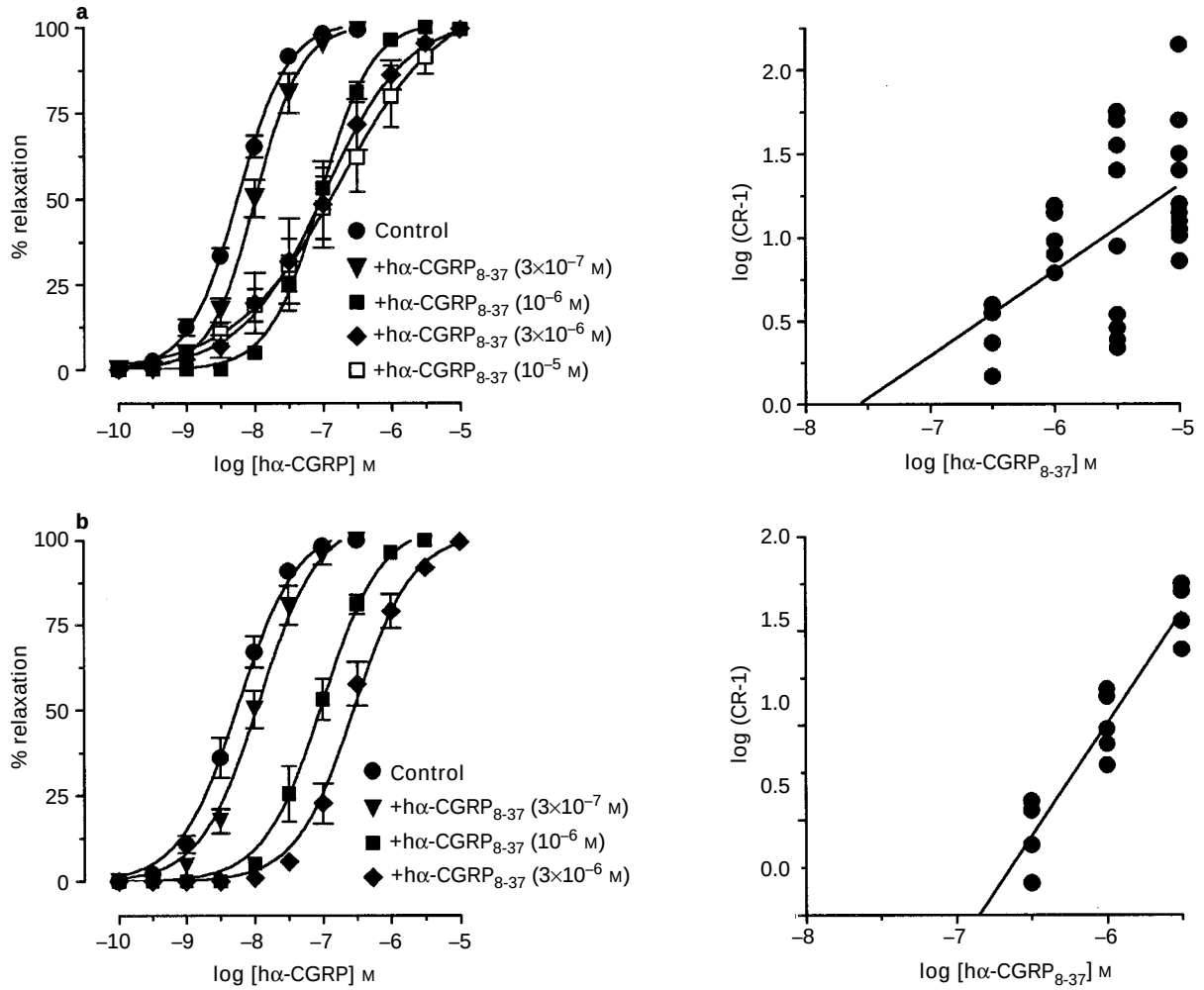

Figure 4 Antagonist activity of hα-CGRP$_{8-37}$ against hα-CGRP in rat pulmonary artery. Graphs (left) showing dose-response curves to hα-CGRP alone (control) on phenylephrine-induced tone, and in the presence of hα-CGRP$_{8-37}$ at 3×10^{-7} M (a and b), 10^{-6} M (a and b), 3×10^{-6} M (a and b) and 10^{-5} M (a). Results are expressed as percentage relaxation of the spasmogen-induced tone, where points represent the mean and vertical lines show s.e.mean of 4 to 28 individual experiments. Where error bars are not shown they are smaller than the symbols. The Schild plots (right) for hα-CGRP$_{8-37}$ against hα-CGRP with (a) a shallow slope and (b) a slope not significantly different from unity (see text). Points represent individual values from at least 13 experiments.

dependent parallel rightward shift of the agonist curve (Figure 4a). Construction of a Schild plot gave an apparent pA_2 value of 7.5 with a slope (0.5 ± 0.2), that was significantly different from unity. However, high concentrations of hα-$CGRP_{8-37}$ (e.g. 10^{-5} M) caused relaxation of the spasmogen-induced tone (see above), and this relaxation was associated with a reduced antagonist affinity for hα-$CGRP_{8-37}$, when measured against hα-CGRP (Figure 5). Thus, subsequent results were obtained from the three lowest concentrations of hα-$CGRP_{8-37}$ where either the contractile tone was unaltered (3×10^{-7} and 10^{-6} M), or, with hα-$CGRP_{8-37}$ 3×10^{-6} M, including only those tissues which were not relaxed by the antagonist (Figure 5). With only these sets of results hα-$CGRP_{8-37}$ (3×10^{-7}–3×10^{-6} M) inhibited hα-CGRP responses dose-dependently, and shifted the agonist curve to the right in a parallel manner

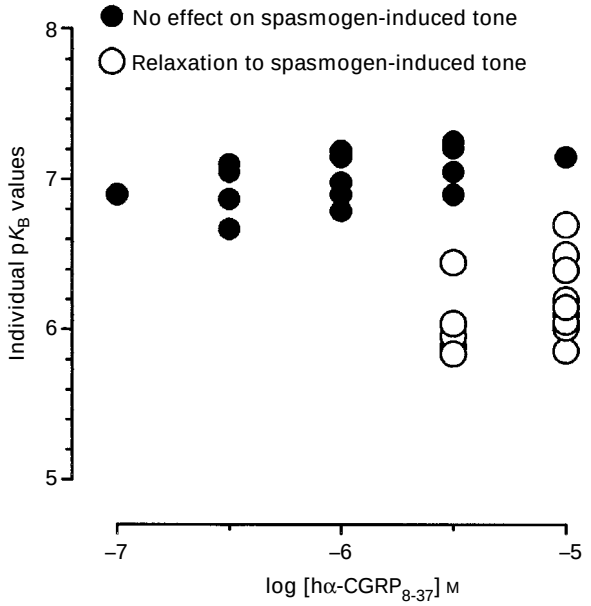

Figure 5 Summary of individual pK_B values for hα-$CGRP_{8-37}$ (10^{-7}–10^{-5} M) against hα-CGRP in rat pulmonary artery. Apparent pK_B values for hα-$CGRP_{8-37}$ were obtained from tissues where addition of the fragment either had no effect on phenylephrine-induced tone or had caused a 20–50% relaxation of the spasmogen-induced tone. Results are expressed as individual pK_B values for hα-$CGRP_{8-37}$, where points represent single values from 29 experiments.

(pA_2 6.9, slope 1.2 ± 0.1; Figure 4b). Subsequently in the pulmonary artery CGRP antagonists were used at 10^{-6} M or lower concentrations, which had no effect on either the basal or the spasmogen-evoked tone.

Human α-$CGRP_{8-37}$ (10^{-6} M) antagonized responses to hβ-CGRP without a reduction in the maximum effect (apparent pK_B 7.1 ± 0.1; Figure 6a), similar to that against hα-CGRP. In contrast, responses to human adrenomedullin were not antagonized by hα-$CGRP_{8-37}$ (10^{-6} M; Figure 6b). The β-form of human $CGRP_{8-37}$ (10^{-6} M) antagonized responses to hα-CGRP (apparent pK_B 7.1 ± 0.1; Figure 6c).

Antagonism by $CGRP_{8-37}$ in the vas deferens

Addition of hα-$CGRP_{8-37}$ (up to 3×10^{-5} M) or hβ-$CGRP_{8-37}$ (up to 10^{-4} M) did not alter either basal tone or twitch responses in the prostatic vas deferens. Incubation of hα-$CGRP_{8-37}$ (10^{-5} M) for either 3, 20 or 60 min before addition of hα-CGRP (apparent pK_B 6.1 ± 0.1, 6.0 ± 0.2, <5.0 respectively, $n=4$ each), showed that equilibrium was reached after 3 min. Thereafter, CGRP fragments were incubated for 20 min before addition of agonists.

Pretreatment with hα-$CGRP_{8-37}$ at concentrations up to 3×10^{-6} M did not significantly alter responses to hα-CGRP, ($P > 0.05$; data not shown). However, higher concentrations of hα-$CGRP_{8-37}$ (10^{-5}–3×10^{-5} M) assayed against hα-CGRP responses, produced a parallel rightward shift of the agonist curve (pA_2 6.0, slope 0.9 ± 0.1; Figure 7a; Table 2). Human α-$CGRP_{8-37}$ antagonized hβ-CGRP, rat β-CGRP and rat amylin responses with an affinity similar to that obtained against hα-CGRP (Figure 7b and 8; Table 2). The β-form of human $CGRP_{8-37}$ (3×10^{-5}–10^{-4} M) dose-dependently antagonized hα-CGRP responses with a similar affinity to hα-$CGRP_{8-37}$ (Figure 7c; Table 2).

In experiments where contractile responses were produced by addition of noradrenaline (10^{-5} M) in the epididymal and prostatic vas, hα-$CGRP_{8-37}$ (10^{-6}–10^{-5} M) assayed against hα-CGRP gave pA_2 values which were similar to those found in field-stimulated preparations (Table 2).

Effect of peptidase inhibitors

In the pulmonary artery peptidase inhibitors (10^{-6} M of amastatin, bestatin, captopril, phosphoramidon, thiorphan;

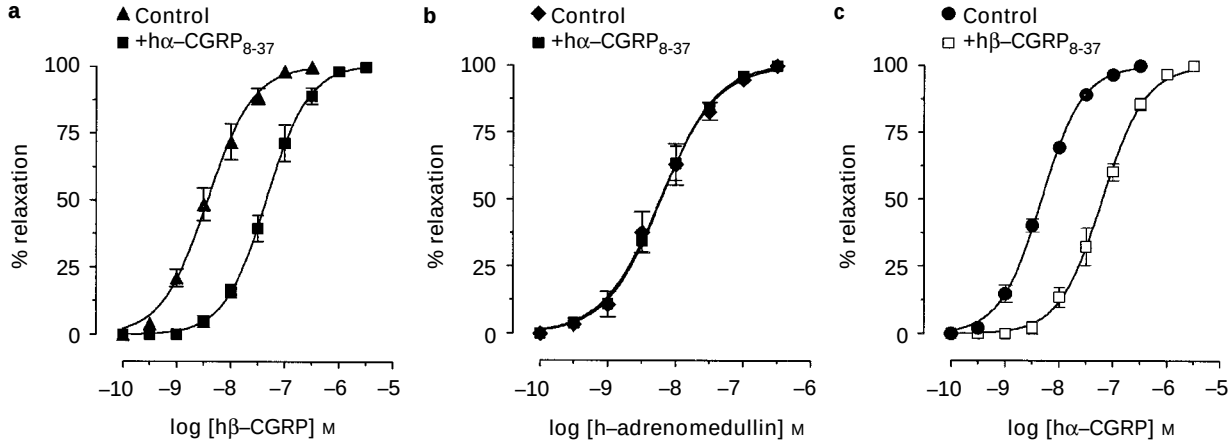

Figure 6 Effect of hα-$CGRP_{8-37}$ on hβ-CGRP and adrenomedullin responses and effect of hβ-$CGRP_{8-37}$ on hα-CGRP responses in rat pulmonary artery. Dose-response curves to (a) hβ-CGRP, (b) human adrenomedullin, (c) hα-CGRP (control) on phenylephrine-induced tone, and after pretreatment with hα-$CGRP_{8-37}$ (10^{-6} M; a and b) and hβ-$CGRP_{8-37}$ (10^{-6} M; c). Results are expressed as % relaxation of the spasmogen-induced tone. Points represent the mean and vertical lines show s.e.mean of 4 experiments. Where the error bars are not shown they are smaller than the symbols.

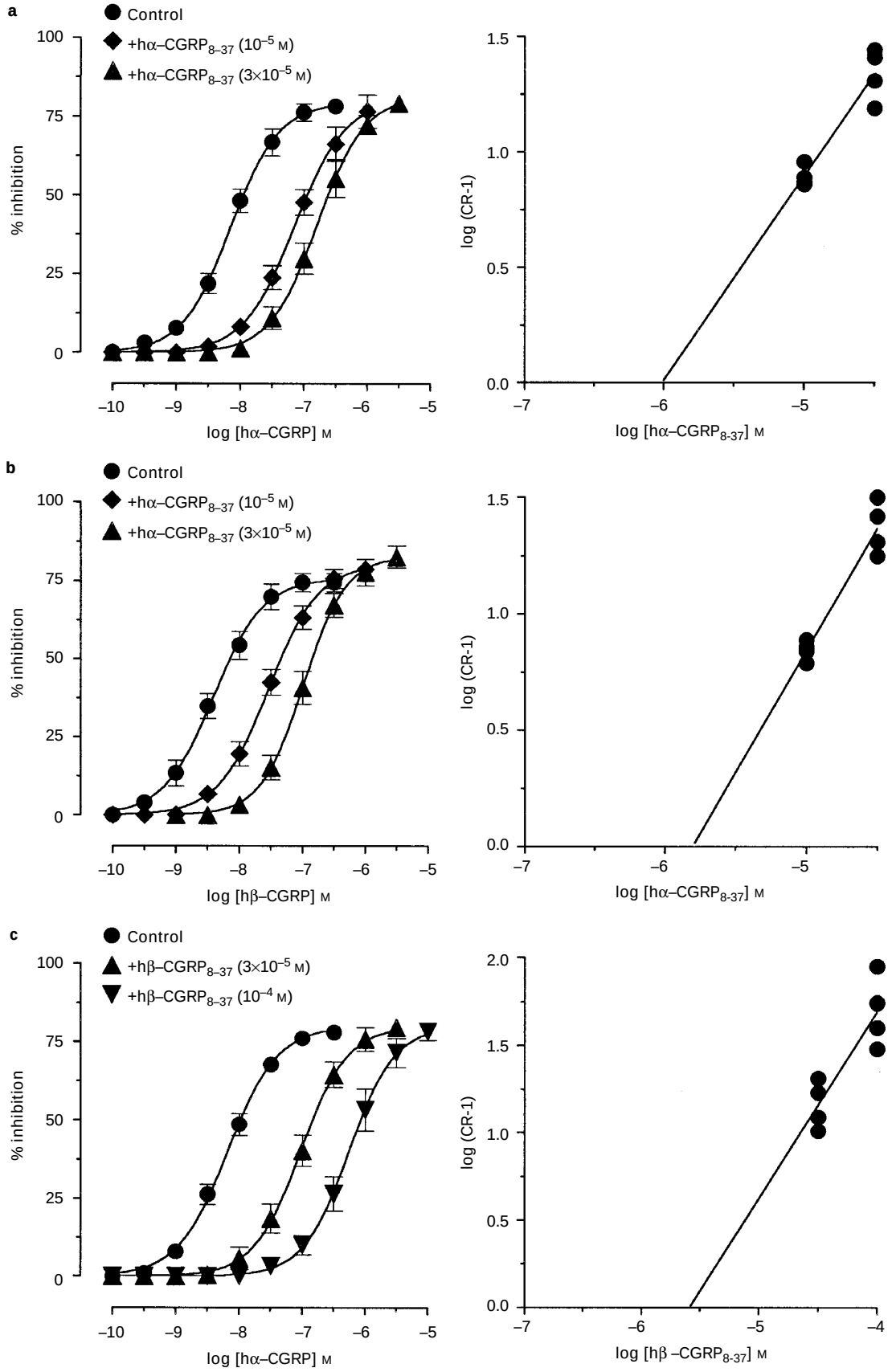

Figure 7 Antagonism by hα-CGRP$_{8-37}$ of the effects of hα- and *β*-CGRP and by hβ-CGRP$_{8-37}$ of those of hα-CGRP in rat prostatic vas deferens. Graphs (left) showing dose response curves to (a and c) hα-CGRP and (b) hβ-CGRP (control) on twitch responses, and in the presence of hα-CGRP$_{8-37}$ (10^{-5} M and 3×10^{-5} M; a and b) and hβ-CGRP$_{8-37}$ (3×10^{-5} M and 10^{-4} M; c). Results are expressed as percentage inhibition of twitch responses. Points represent the mean and vertical lines show s.e.mean of 4 to 8 experiments. The Schild plots for hα-CGRP$_{8-37}$ against (a) hα-CGRP, (b) hβ-CGRP and for (c) hβ CGRP$_{8-37}$ against hα-CGRP are shown on the right. Points represent individual values from 8 experiments.

Table 2 Antagonist affinities of hα-$CGRP_{8-37}$ and hβ-$CGRP_{8-37}$ against CGRP analogues on inhibition of either twitch responses or noradrenaline-induced tone in the rat prostatic and epididymal vas deferens

Preparation	*Agonist*	*Antagonist*	*pA_2/pK_B* value*	*Schild slope*
Prostatic VD (EFS)	hα-CGRP	hα-$CGRP_{8-37}$	6.0	0.9±0.1
Prostatic VD (NA)	hα-CGRP	hα-$CGRP_{8-37}$	5.8	1.0±0.2
Epididymal VD (NA)	hα-CGRP	hα-$CGRP_{8-37}$	5.8	1.0±0.3
Prostatic VD (EFS)	hβ-CGRP	hα-$CGRP_{8-37}$	5.7	1.2±0.1
Prostatic VD (EFS)	Rat β-CGRP	hα-$CGRP_{8-37}$ (10^{-5} M)	5.8±0.1*	–
Prostatic VD (EFS)	Rat amylin	hα-$CGRP_{8-37}$ (10^{-5} M)	5.8±0.1*	–
Prostatic VD (EFS)	hα-CGRP	hβ-$CGRP_{8-37}$	5.6	1.1±0.2

pK_B values (*) were obtained from concentration-ratios produced by the stated concentration of the CGRP antagonists, where values are expressed as mean±s.e.mean. pA_2 values were obtained from a Schild plot by linear regression with various concentrations of the CGRP antagonists, where the Schild slope was expressed as mean±s.e.mean. Results were obtained from at least 4 experiments. Contractile responses in the prostatic and epididymal portions of the vas deferens (VD) were either evoked by electrical field stimulation (EFS) or by addition of 10^{-5} M noradrenaline (NA).

30 min pretreatment) did not significantly alter the effect of hα-CGRP or hα-$CGRP_{8-37}$ ($P>0.05$; pEC_{50} hα-CGRP 8.1±0.1 and 8.0±0.1 ($n=4$ each), and apparent pK_B for hα-$CGRP_{8-37}$ (10^{-6} M) against hα-CGRP 7.0±0.1 and 6.9±0.1 ($n=4$ each), in the absence and presence of peptidase inhibitors, respectively).

In the prostatic vas deferens, peptidase inhibitors did not significantly modify the agonist activity of either hα-CGRP or [Cys($ACM^{2,7}$)] hα-CGRP ($P>0.05$; pIC_{50} for hα-CGRP 7.8±0.1 and 7.6±0.1 ($n=4$ each), and the maximum responses for 3×10^{-4} M [Cys($ACM^{2,7}$)] hα-CGRP were 45.0±5.6% and 39.3±4.5%, in the absence and presence of peptidase inhibitors, respectively). The affinity of hα-$CGRP_{8-37}$ (10^{-5} M) assessed against hα-CGRP responses in the vas was not changed in the presence of peptidase inhibitors ($P>0.05$; apparent pK_B hα-$CGRP_{8-37}$ 5.9±0.1 and 5.8±0.1 ($n=4$ each) in the absence and presence of peptidase inhibitors, respectively). Thus inhibition of peptidases did not increase either the potency of CGRP agonists or the affinity of CGRP antagonists in these rat tissues.

Discussion

The proposed classification into $CGRP_1$ and $CGRP_2$ receptors has been largely dependent on the C-terminal fragment hα-$CGRP_{8-37}$, which has a higher affinity at the $CGRP_1$ receptor than at the $CGRP_2$ receptor (Dennis *et al*., 1989; 1990; Mimeault *et al*., 1991; Quirion *et al*., 1992). The pA_2 value for hα-$CGRP_{8-37}$ (6.9 against hα-CGRP) in the rat pulmonary artery is consistent with a $CGRP_1$ receptor, and in good agreement with pA_2 values (6.9–7.7) obtained from the prototypical $CGRP_1$ receptor in the guinea-pig atrium (Dennis *et al*., 1989; Maggi *et al*., 1991; Mimeault *et al*., 1991). In the rat vas deferens, the lower pA_2 value of 6.0 for hα-$CGRP_{8-37}$ is consistent with a $CGRP_2$-receptor, and agrees well with values from the literature in this tissue, ranging from 6.6 to less than 6.0 (Dennis *et al*., 1990; Maggi *et al*., 1991; Mimeault *et al*., 1991; 1992; Longmore *et al*., 1994). Therefore, the differing affinities for hα-$CGRP_{8-37}$ in these rat tissues support the proposed CGRP receptor classification, in a single species. The observation that hα-$CGRP_{8-37}$ antagonizes the effect of different CGRP forms in an agonist-independent manner, suggests that the rat pulmonary artery and vas deferens contain a single class of $CGRP_1$ and $CGRP_2$ receptors, respectively. These receptor subtypes can also be distinguished by hβ-$CGRP_{8-37}$, which shows the same difference in antagonist affinity as the human α-form. In the vas deferens, contractions either through field stimulation or a spasmogen (noradrenaline) or differences between the parts of the vas did not alter the antagonist affinity for hα-$CGRP_{8-37}$, suggesting that both epididymal and prostatic portions contain a common population of $CGRP_2$ receptors. Thus as sequence differences between the CGRP agonists and antagonists do not lead to differences in selectivity for the receptor subtypes this supports the characterization of rat $CGRP_1$ and $CGRP_2$ receptors. Further, since equilibrium appeared to have been reached for hα-$CGRP_{8-37}$ at both receptors this is not a factor which could be responsible for the differing affinities. However, the present conclusions are based on an around 10 fold difference in $CGRP_{8-37}$ affinity, which clearly indicates the need for antagonists with greater selectivity.

Recently, Longmore *et al*. (1994) suggested that differing $CGRP_{8-37}$ affinities may reflect differences in enzyme distribution, as the peptidase inhibitor thiorphan (10^{-5} M) increased the affinity to hα-$CGRP_{8-37}$ in the rat vas (from an apparent pK_B value of less than 6.0 to a value of 6.6). However, the present study indicates that peptide degradation does not account for the differing affinities, since neither the potency of hα-CGRP nor the affinity for hα-$CGRP_{8-37}$ was potentiated in the presence of several peptidase inhibitors. Furthermore, previous work in the rat vas deferens performed in the presence of thiorphan (Giuliani *et al*., 1992) gave apparent pK_B values for hα-$CGRP_{8-37}$ not different from the present results. Therefore, differences in peptide degradation do not explain the difference in hα-$CGRP_{8-37}$ affinity between the rat vas and pulmonary artery.

The linear analogue [Cys($ACM^{2,7}$)] hα-CGRP has been suggested as a selective agonist at the $CGRP_2$ receptor in the rat vas (1% potency relative to hα-CGRP), while being inactive at the $CGRP_1$ receptor (up to 10^{-6} M; Dennis *et al*., 1989; 1990; Mimeault *et al*., 1991; Quirion *et al*., 1992). The present results in the rat vas are not in agreement with this proposal since [Cys($ACM^{2,7}$)] hα-CGRP was at least 3000 fold weaker in activity than hα-CGRP, although the potency of other agonists such as (human and rat) CGRP or rat amylin was similar to

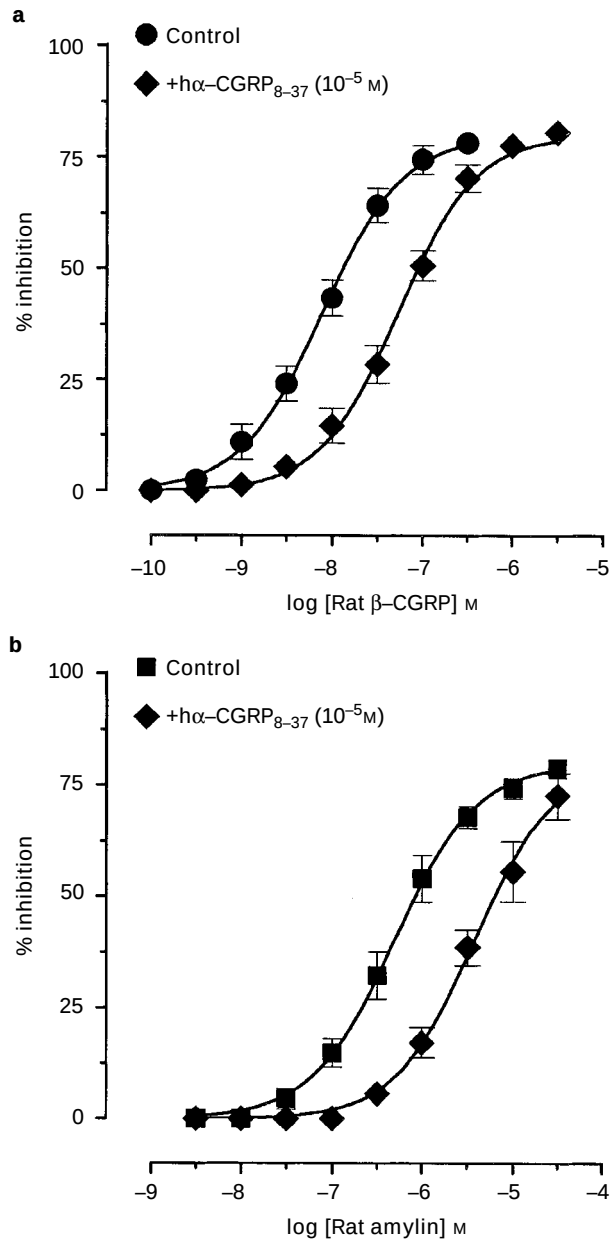

Figure 8 Antagonist effect of hα-$CGRP_{8-37}$ against rat β-CGRP and rat amylin in rat prostatic vas deferens. Dose-response curves for effects of (a) rat β-CGRP and (b) rat amylin alone (control) on twitch responses, and after pretreatment with hα-$CGRP_{8-37}$ (10^{-5} M). Results are expressed as percentage inhibition of twitch responses. Points represent the mean and vertical lines show s.e.mean of 4 experiments.

those found by other workers (Dennis *et al*., 1989; Maggi *et al*., 1991; Giuliani *et al*., 1992). The weak activity of [$Cys(ACM^{2,7})$] hα-CGRP might reflect metabolism but the lack of effect of the peptidase inhibitors makes this unlikely. Another possibility may be that the peptide acts as a partial agonist, reflecting receptor reserve. It is also noteworthy that there was no confirmation of the proposed 1% relative potency of [$Cys(ACM^{2,7})$] hα-CGRP (compared to hα-CGRP) at a $CGRP_2$ receptor and studies which have suggested $CGRP_1$ receptors on the basis of [$Cys(ACM^{2,7})$] hα-CGRP inactivity (Claing *et al*., 1992; Chin *et al*., 1994) did not use higher concentrations than 10^{-6} M, despite the fact that the peptide is only a weak agonist. Therefore, at least based on the present findings, it appears that [$Cys(ACM^{2,7})$] hα-CGRP does not discriminate between the proposed $CGRP_1$ and $CGRP_2$ receptors (being at least 3000 fold less active than hα-CGRP at both receptors).

Recently, Giuliani *et al*. (1992) suggested that amylin might differentiate between $CGRP_1$ and $CGRP_2$ receptors, since hα-$CGRP_{8-37}$ antagonized rat amylin responses in the guinea-pig atrium but not in the rat vas deferens. However, present results in the rat vas suggest that rat amylin has affinity for the $CGRP_2$ receptor, since hα-$CGRP_{8-37}$ had the same apparent affinity against amylin as against CGRP, consistent with a homogenous population of $CGRP_2$ receptors that is activated by both types of peptides. Furthermore, amylin does not consistently mimic the effect of CGRP on $CGRP_1$ receptors (Tomsinlon & Poyner, 1996). Therefore, the use of amylin to differentiate CGRP receptors appears to be of limited value.

Human adrenomedullin does not act via $CGRP_1$ receptors in the pulmonary artery, since it was not antagonized by hα-$CGRP_{8-37}$, which is in agreement with several studies (e.g. rat perfused lung; Heaton *et al*., 1995). Therefore, the results from the rat pulmonary artery are consistent with the existence of separate adrenomedullin and $CGRP_1$ receptors, which can be differentiated by hα-$CGRP_{8-37}$.

CGRP acts in the rat pulmonary artery by an endothelium-dependent mechanism via nitric oxide, as responses were abolished either by removal of the endothelium or with N^G-nitro-L-arginine (which could be reversed with L-arginine). This conclusion is consistent with previous binding and *in vitro* studies (Mannan *et al*., 1995), and agrees well with the mechanism found in, for instance, the rat thoracic aorta (Gray & Marshall, 1992a,b).

The affinity of hα-$CGRP_{8-37}$ in the rat pulmonary artery agrees with several vascular studies, including the rat perfused mesenteric vasculature (pA_2 7.4 against rat α-CGRP; Nuki *et al*., 1994), rat mesenteric resistance artery (apparent pK_B 7.0 (10^{-6} M) against hβ-CGRP; Lei *et al*., 1994), guinea-pig superior mesenteric artery (apparent pK_B around 6.95 (10^{-6} M) against human CGRP; Gyoda *et al*., 1995) and pig coronary artery (pA_2 6.7 against hα-CGRP; Gray *et al*., 1991). However, these studies reflect an endothelium-independent pathway for CGRP (via $CGRP_1$ receptors), whereas the receptor in the pulmonary artery is an endothelial $CGRP_1$ receptor. Thus receptors with higher affinity for hα-$CGRP_{8-37}$ can be associated with the endothelium and not just with the vascular smooth muscle. In the vas deferens, CGRP receptors are located on the smooth muscle, since inhibition of twitch responses by CGRP has been shown to be a direct effect through postjunctional receptors and is not neuronally mediated (Al-Kazwini *et al*., 1986; Goto *et al*., 1987).

While $CGRP_{8-37}$ may be the most reliable tool to subclassify CGRP receptors, there are problems with this antagonist. Firstly, its affinity was reduced with longer incubation times (60 min) in the rat vas but not in the pulmonary artery, which might reflect the uptake of hα-$CGRP_{8-37}$ in some tissues. Secondly, in the pulmonary artery, the Schild analysis for hα-$CGRP_{8-37}$ gave a regression with a slope less than unity (when all concentrations of hα-$CGRP_{8-37}$ were included). The possibility that CGRP receptors are present on the smooth muscle activated by high concentrations of CGRP is unlikely, since hα-CGRP up to 10^{-5} M gave no response in the absence of the endothelium, although other explanations for a 'nonequilibrium' steady state could be possible. The observation that the antagonist hα-$CGRP_{8-37}$ had vasodilator activity might suggest that the peptide is a partial agonist in the pulmonary artery. No relaxation was seen in the vas deferens with antagonist concentrations up to 10^{-4} M (hβ-$CGRP_{8-37}$) suggesting a difference between the two receptor subtypes. Alternatively, it may be possible that hα-$CGRP_{8-37}$ acts at a

non-CGRP receptor in the pulmonary artery which is not present in the vas. Therefore, the present results indicate that the fragment has to be used with care in some situations, since there are limitations in its usefulness as an antagonist.

In conclusion, the difference in affinity of $CGRP_{8-37}$ supports the proposed classification of $CGRP_1$ and $CGRP_2$ receptors in a single species, in the rat pulmonary artery and vas deferens, respectively. However, the CGRP analogue [Cys($ACM^{2,7}$)] hα-CGRP and the homologues amylin and adrenomedullin appear not to be useful pharmacological criteria to distinguish between the putative CGRP receptor subtypes.

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