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Involvement of multiple receptors in the biological effects of calcitonin gene-related peptide and amylin in rat and guinea-pig preparations

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1 The activity of rat α and β calcitonin gene-related peptide (CGRP) as compared to the structurally related peptide, rat amylin, has been investigated in the guinea-pig isolated left atrium (electrically driven), in mucosa-free strips from the base of the guinea-pig urinary bladder and in the rat isolated vas deferens (pars prostatica). The antagonist activity of the C-terminal fragment of human αCGRP, αCGRP(8-37), was also investigated.

2 In the guinea-pig isolated left atrium the three peptides produced a concentration-related positive inotropic effect, amylin being about 16 and 31 times less potent than α or βCGRP, respectively. Human αCGRP(8-37) produced a rightward displacement of the log concentration-response curve to the three agonists tested, without depression of maximal response attainable. Apparent pK_B values calculated on the basis of the displacement produced by 1 μM human αCGRP(8-37) indicated an agonist-independent affinity of the antagonist (6.66 ± 0.11 for αCGRP, 6.42 ± 0.17 for βCGRP and 6.95 ± 0.11 for amylin).

3 In the guinea-pig isolated urinary bladder, α or βCGRP or amylin produced a concentration-related inhibition of twitch contractions evoked by train electrical field stimulation (10 Hz frequency, 0.25 ms duration at 100 V for 0.5 s every 60 s). Amylin was about 100 times less potent than α or βCGRP. Human αCGRP(8-37) (3 μM) did not significantly affect the inhibitory action of the three agonists tested.

4 In the rat isolated vas deferens, α or βCGRP or amylin produced a concentration-related inhibition of twitch contractions evoked by electrical field stimulation (0.2 Hz frequency, 0.5 ms duration at 60 volts). Amylin was about 100 times less potent than α or βCGRP. Human αCGRP(8-37) at 3 μM did not significantly affect the inhibitory action of amylin and at 3 μM antagonized the responses to rat α and βCGRP with apparent pK_B values of 5.86 ± 0.15 and 6.11 ± 0.13, respectively.

5 These findings indicate that multiple receptors mediate the actions of peptides of the CGRP/amylin family in the preparations investigated. In the guinea-pig atrium both α and β forms of rat CGRP as well as amylin act by stimulating a single class of receptors which are sensitive to the inhibitory action of human αCGRP(8-37). In rat isolated vas deferens, at least two receptors could be present, one activated by α and βCGRP and partially sensitive to human αCGRP(8-37) and another which is sensitive to amylin but not recognised by human αCGRP(8-37). This latter type of receptor could be entirely responsible for the action of the agonists in the guinea-pig urinary bladder.

Keywords: Calcitonin gene-related peptide; CGRP receptors; rat α and β-CGRP; rat amylin; CGRP antagonist

Introduction

Calcitonin gene-related peptide (CGRP) is a 37 amino acid residue neurotransmitter peptide widely distributed in the central and peripheral nervous system in mammals (Yamamoto & Tohyama, 1989, for review). CGRP is produced through an alternate splicing of the calcitonin gene and is the predominant form expressed in the neuronal tissue. Various molecular forms of CGRP have been described in various species, which produce their biological actions through specific receptors expressed on the membrane of target cells (see Breimer *et al.*, 1988, for review). Two forms of CGRP, termed α and β are produced in both rat and man which differ in a few residues only. Both α and β forms of CGRP are endowed with biological activity in various assays including powerful vasodilator, cardiac positive inotropic and smooth muscle relaxant activities. Recently, it has been discovered that the C-terminal fragment, human αCGRP(8-37), binds with relatively high affinity to CGRP receptors but does not possess intrinsic activity to stimulate the biological response (Chiba *et al.*, 1989). According to these biochemical observations, human αCGRP(8-37) acts as a competitive antagonist at certain CGRP receptors (e.g. Dennis *et al.*, 1990; Maggi *et al.*, 1991). The use of these fragments has been instrumental in proving definitively the neurotransmitter role of CGRP in e.g. nerve-mediated responses in the guinea-pig atrium (Maggi *et al.*, 1991), guinea-pig ileum (Bartho *et al.*, 1991), guinea-pig ureter and renal pelvis (Maggi & Giuliani, 1991; Maggi *et al.*, 1992) and rat vas deferens (Maggi *et al.*, 1991).

Owing to various pharmacological criteria, including the different potency of human CGRP(8-37) in antagonizing the actions of CGRP in various biassays, the proposal has been advanced (Dennis *et al.*, 1989; 1990; Mimeault *et al.*, 1991; Quirion *et al.*, 1992) that two subtypes of CGRP receptors may exist, which have been termed $CGRP_1$ and $CGRP_2$. It has been proposed that certain preparations such as the guinea-pig atrium are rich in $CGRP_1$ receptors, while others, such as the rat vas deferens preferentially express $CGRP_2$ receptors (Dennis *et al.*, 1989; 1990). The existence of multiple receptors raises the question as to whether the various forms of endogenous CGRP may have preferential affinity for the various receptor subtypes, as has been demonstrated with peptides of other families. The first aim of this study was to compare the action of the α and β forms of rat CGRP in the guinea-pig isolated atrium and rat vas deferens. The

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guinea-pig isolated bladder was also included in the study because it is a sensitive bioassay for CGRP (Maggi *et al.*, 1988) and comparison with the data obtained in the atrium may indicate whether CGRP receptors in different organs of the same species have different pharmacology.

Amylin, also known as islet amyloid peptide, is a 37 amino acid residue peptide originally isolated from amyloid plaques in the pancreas of patients with non-insulin-dependent diabetes mellitus (Westermark *et al.*, 1987; Cooper *et al.*, 1987). Amylin is produced from a distinct gene and mature amylin shares about 50% homology with CGRP peptides: the genes encoding amylin and CGRP/calcitonin are believed to belong to a superfamily derived by duplication of a common ancestral gene (Cooper *et al.*, 1989). Interestingly, amylin and CGRP/calcitonin peptides share some biological activities such as regulation of glycogen metabolism in smooth muscles (Leighton & Cooper, 1988), calcium metabolism (Datta *et al.*, 1989) and haemodynamic actions (Brain *et al.*, 1990; Gardiner *et al.*, 1991). The haemodynamic actions produced by amylin infusion in rats are blocked by human αCGRP(8-37) suggesting stimulation of a common population of receptors (Gardiner *et al.*, 1991). CGRP and amylin have been shown to interact at the same receptor sites in rat liver and skeletal muscle membranes by use of ^{125}I-labelled human αCGRP as ligand (Chantry *et al.*, 1991), while a recent report (Poyner *et al.*, 1992) failed to indicate a substantial interaction of amylin with CGRP receptors on rat L6 myocytes. We have therefore included amylin in the study and have investigated the ability of human CGRP(8-37) to prevent the action of this peptide.

Methods

Male albino guinea-pigs (250–300 g) and male albino rats of Wistar strain (300–340 g) were stunned and bled.

For experiments on the guinea-pig atrium the animals received reserpine (5 mg kg^{-1}, i.p., 48–96 h before the experiment). Reserpine pretreatment was found to enhance the response to exogenous CGRP and improve reproducibility of concentration-response curves to CGRP in this preparation (Giuliani, unpublished data). The guinea-pig left atrium was quickly removed and placed in oxygenated Tyrode solution containing atropine (1 μM) and prepared for isometric tension recording, as described previously (Giuliani *et al.*, 1989; 1991; Maggi *et al.*, 1991). The atria were electrically driven at a frequency of 3 Hz (0.5 ms duration at maximal voltage) in order to obtain a stable baseline inotropic activity. Addition of human αCGRP produced concentration-dependent positive inotropic effect suitable for assessing CGRP receptor antagonism (Maggi *et al.*, 1991).

The guinea-pig urinary bladder and rat vas deferens (pars prostatica) were excised and placed in oxygenated Krebs solution. A mucosa-free strip of smooth muscle was prepared from the base of the guinea-pig urinary bladder as described previously (Maggi *et al.*, 1988). A 10 mm segment of the rat vas deferens or the muscle strip from the guinea-pig bladder was prepared for isometric tension recording under a resting load of 5 mN, as described previously (Maggi *et al.*, 1988).

In each preparation, cumulative concentration-response curves to rat α or βCGRP and amylin were constructed, the next concentration being added when the effect of the preceding one had reached a steady state. Concentrations of amylin higher than 3 μM were not tested because of the limited amount of the peptide. In each preparation, the effect of the three putative agonists was investigated in the absence and the presence of the C-terminal fragment of human αCGRP, CGRP(8-37), which acts as a competitive antagonist at certain CGRP receptors. The effects of the antagonist were determined after having recorded two reproducible concentration-response curves to the agonists. Human αCGRP (8-37) (1–3 μM) was added to the bath 5 min before the beginning of the concentration-response curve to the agonist. All experiments were performed in the presence of 10 μM thiorphan to reduce possible degradation of CGRP by endopeptidases (Le Greves *et al.*, 1989). No agonist effect of human CGRP(8-37) was observed.

Data evaluation

EC_{50} values in the absence and presence of the CGRP receptor antagonist were calculated by linear regression and the least squares method. Apparent pK_B values were calculated from dose-ratios produced by the stated concentration of the CGRP antagonists tested from the equation: $pK_B = \log (x - 1) - \log[\text{antagonist}]$.

Parallelism of the concentration-response curves to CGRP obtained in the presence and absence of various concentrations of antagonists was assessed by use of the Parallel Lines I computer programme described by Tallarida & Murray (1981).

Drugs

Human αCGRP(8-37) (hCGRP(8-37)), rat α and βCGRP and rat amylin were from Peninsula (USA). Stock solutions of the peptides (final concentration 1 mM) were prepared in distilled water and diluted in saline just before use. Other drugs used were: thiorphan (Bachem, Switzerland), atropine and reserpine (Serva, Germany).

Results

Guinea-pig atrium

In the electrically driven guinea-pig left atrium, rat αCGRP produced a concentration-related positive inotropic effect, as described previously (Maggi *et al.*, 1991). Both amylin and rat βCGRP mimicked the action of rat αCGRP and approached the same maximum effect (expressed as % increase in resting inotropism) (Figures 1 and 2, Table 1). Amylin was about 16 and 31 times less potent than α or βCGRP, respectively.

hCGRP(8-37) (1 μM) antagonized the positive inotropic effect of both rat α and βCGRP and amylin in the guinea-pig left atrium, without depressing the maximum response to the agonist (Figure 3). From the dose-ratios obtained in the presence and absence of the antagonist, an apparent pK_B value of 6.66 ± 0.11 ($n = 5$), 6.42 ± 0.17 ($n = 4$) and 6.95 ± 0.11 ($n = 4$) was calculated with rat αCGRP, rat βCGRP or amylin as agonists, respectively.

Guinea-pig urinary bladder

In muscle strips obtained from the guinea-pig isolated urinary bladder, rat αCGRP produced a concentration-related depression of the electrically-evoked (trains of pulses at a frequency of 10 Hz, 0.25 ms duration at 100 volts for 0.5 s every 60 s), tetrodotoxin-sensitive twitch contractions produced by electrical field stimulation (Figure 1). Rat βCGRP was as potent and effective as rat αCGRP in this assay (Figure 2, Table 1). Amylin (30 nM–3 μM) was significantly less potent than the α or β forms of CGRP and, at the highest concentration tested it produced about 70% of the maximal inhibitory effect of CGRP (Figure 2). Amylin was about 100 times less potent than α or β forms of rat CGRP in this assay. Thus, the same degree of inhibition produced by rat αCGRP at 10 nM (36 ± 10% inhibition of twitches) was produced by amylin at 1 μM (37 ± 11%).

Human αCGRP(8-37) (3 μM) was without effect on the inhibitory action produced by rat αCGRP, rat βCGRP or amylin in the guinea-pig urinary bladder (Figure 3).

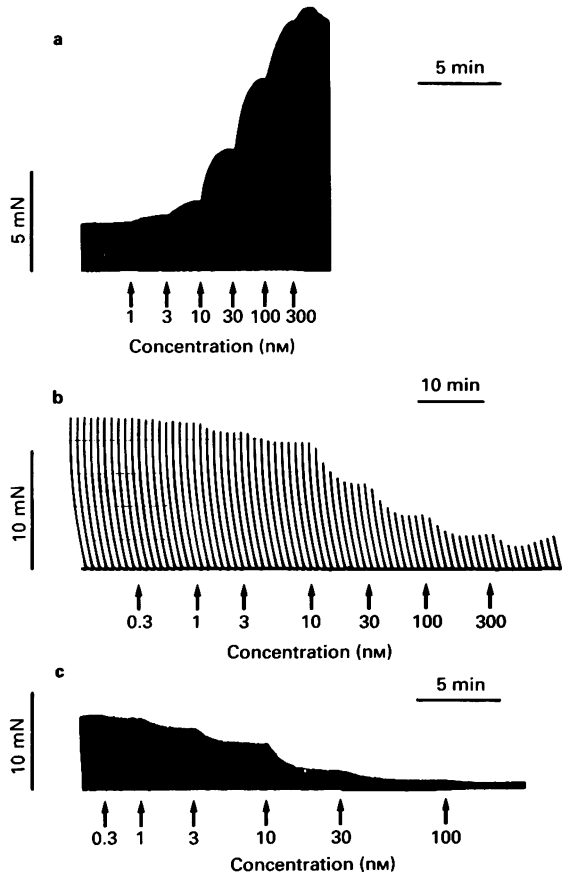

Figure 1 Typical tracings illustrating the concentration-related positive inotropic response to rat α calcitonin gene-related peptide (αCGRP) in the electrically driven guinea-pig isolated left atrium (a), and the concentration-related inhibitory effect of rat αCGRP toward electrically-evoked nerve-mediated contrations of the guinea-pig isolated urinary bladder (b) and rat vas deferens (c).

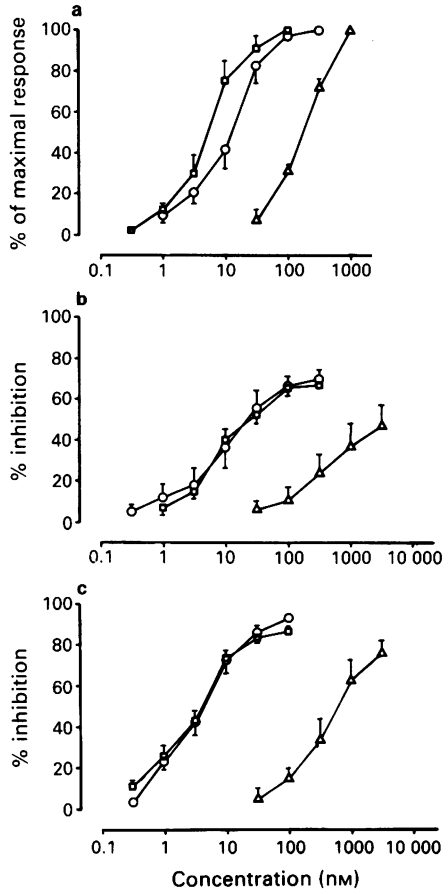

Figure 2 Concentration-related effects of rat α calcitonin gene-related peptide (αCGRP) (○), rat βCGRP (□) and amylin (△) in the guinea-pig left atrium (a) guinea-pig urinary bladder (b) and rat vas deferens (c). Each value is mean with s.e.mean (vertical bars) of 4–5 experiments.

Table 1 EC_{50} (nM) and 95% confidence limits (in parentheses) and maximal effect (E_{max}, expressed as % of the maximal response to rat α calcitonin gene-related peptide (αCGRP)) for rat α or βCGRP and amylin in the guinea-pig atrium, urinary bladder and rat vas deferens

	Rat αCGRP	*Rat βCGRP*	*Amylin*
Guinea-pig atrium			
EC_{50} (nM)	9.9 (6–24)	5.1 (3–11)	158 (65–618)
E_{max}	100	100	100
Guinea-pig urinary bladder			
EC_{50} (nM)	8.0 (5–27)	8.1 (4–66)	NE
E_{max}	100	100	67 ± 6*
Rat vas deferens			
EC_{50} (nM)	4.1 (3–6)	18 (10–38)	NE
E_{max}	100	100	82 ± 5*

Each value is mean of 4–5 experiments.
NE = not evaluable.
*Maximal effect produced at 3 μM.

Rat vas deferens

In the rat isolated vas deferens, rat αCGRP produced a concentration-related inhibition of electrically-evoked (0.2 Hz, 60 V, 0.5 ms pulse width) twitches (Figure 1). Rat βCGRP was as potent and effective as rat αCGRP in this bioassay (Figure 2, Table 1). Amylin was significantly less potent than α or β CGRP and at the highest concentration tested (3 μM) produced 81% of the maximum response to rat αCGRP (Figure 2, Table 1). As observed in the guinea-pig urinary bladder, amylin was about 100 times less potent than the α or β forms of rat CGRP, as judged by the concentration of the various agonists required to produce a similar submaximal inhibitory effect (Figure 2).

Human αCGRP(8-37) (3 μM) produced a rightward displacement of the concentration-response curve to rat α and βCGRP, without producing depression of the maximum response attainable. From the dose-ratios obtained in the presence and absence of antagonist, apparent pK_B values of 5.86 ± 0.15 and 6.11 ± 0.13 were calculated for human αCGRP (8-37) against rat α and βCGRP, respectively (Figure 3).

Human αCGRP(8-37) did not significantly affect the inhibitory action of rat amylin in the rat isolated vas deferens (Figure 3).

Discussion

The present findings provide further evidence suggesting a heterogeneity of CGRP receptors in rat and guinea-pig tissue. Furthermore, evidence is presented indicating that amylin may act as an agonist at certain CGRP receptors such as those present in the guinea-pig left atrium. The studies of Quirion and coworkers (Dennis *et al.*, 1989; 1990; Mimeault *et al.*, 1991), summarized by Quirion *et al.* (1992) have provided evidence for the existence of two subtypes of CGRP receptor, which have been termed $CGRP_1$ and $CGRP_2$. $CGRP_1$ receptors, for which the guinea-pig atrium is a prototypical assay, are recognized with relatively high affinity by C-terminal fragments of human αCGRP such as CGRP(8-37) or CGRP(12-37). $CGRP_2$ receptors, for which the rat vas

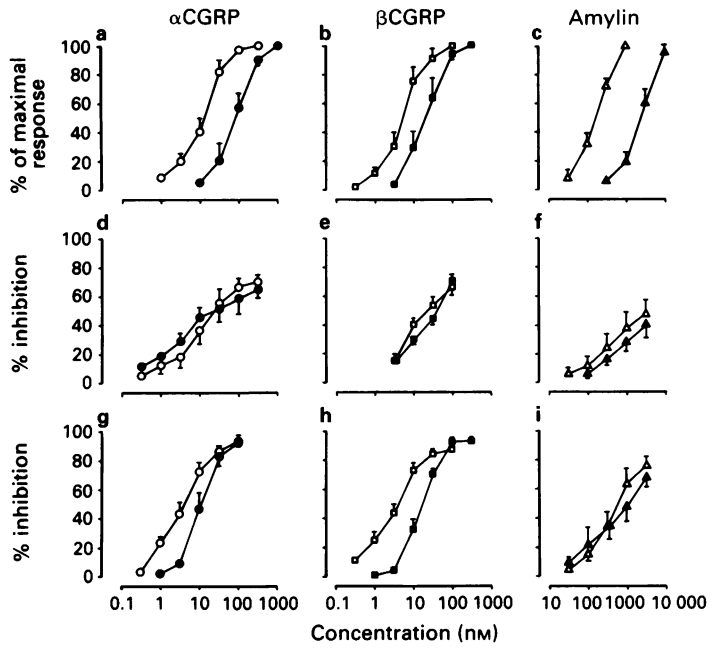

Figure 3 Effect of human α calcitonin gene-related peptide(8-37) (αCGRP(8-37)) on the response to rat αCGRP, rat βCGRP and amylin in the guinea-pig left atrium (a,b,c), guinea-pig urinary bladder (d,e,f) and rat vas deferens (g,h,i). In each panel the effects of peptide alone (control) (open symbols), and in the presence of human αCGRP(8-37) (closed symbols) is shown. Concentration of human αCGRP(8-37) is 1 μM in the atrium, and 3 μM in the other two preparations. Each value is mean with s.e.mean (vertical bars) of 4–5 preparations.

deferens is a prototypical assay, are recognized with lower affinity by CGRP(8-37) while CGRP(12-37) seems inactive as an antagonist at these sites (Mimeault *et al.*, 1991). Thus the ability of human CGRP(8-37) to block the action of the agonist seems crucial to discriminate between the proposed $CGRP_1$ and $CGRP_2$ receptor subtypes: such a discriminating ability of the antagonist has been demonstrated also in *in vivo* assays involving the hypophagic and hypothermic response to central administration of human αCGRP in rats (Quirion *et al.*, 1992) and the gastric antisecretory and antiulcer activity in the same species (Evangelista *et al.*, 1992).

Human αCGRP(8-37) competitively interacts with CGRP binding sites without stimulating them (Chiba *et al.*, 1989). Its competitive antagonist properties against exogenously administered CGRP have been repeatedly demonstrated (see Introduction for References) whilst no inhibitory action toward other agonists (bradykinin, histamine, substance P, vasoactive intestinal polypeptide, isoprenaline, adrenaline or neurotensin) has been reported in various experimental test objects (Donoso *et al.*, 1990; Maggi *et al.*, 1991; Barthó *et al.*, 1991; Chakder & Rattan, 1991). The present findings confirm a somewhat greater affinity of human αCGRP(8-37) at CGRP receptors in the guinea-pig left atrium vs. CGRP receptors in the rat vas deferens and extend the investigations to two other main questions: (a) do the α and β forms of CGRP act on the same receptor population and (b), does amylin interact with the CGRP receptor? Our results indicate that in the guinea-pig left atrium, the α and β forms of rat CGRP, as well as amylin, interact with a single class of CGRP receptors. In fact the agonist action of the three peptides was antagonized by human αCGRP(8-37) in an agonist-independent manner, indicative of a common site of action.

In the rat vas deferens, the action of amylin was not antagonized by hCGRP(8-37); in agreement with the $CGRP_1$ and $CGRP_2$ receptor hypothesis (see above), hCGRP(8-37) was weakly active against rat α or βCGRP, with lower affinity as compared to the guinea-pig atrium. The present findings may be interpreted as indicating that both $CGRP_1$ and $CGRP_2$ sites are present in the vas deferens. Amylin could be acting preferentially at the $CGRP_2$ sites while rat α and βCGRP could act on both sites, thus accounting for the lower potency of hCGRP(8-37) as an antagonist in this assay. According to this interpretation, the guinea-pig urinary bladder base may contain a pure population of the putative $CGRP_2$ receptor sites. In fact human CGRP(8-37) failed to antagonize the action of the three agonists at this level. The role of amylin as a CGRP receptor agonist could be interpreted in the same way: amylin may act as an agonist at both $CGRP_1$ and $CGRP_2$ receptors, although with different affinities. Alternatively, the possibility that amylin is acting, in the guinea-pig urinary bladder and rat vas deferens, at other receptors distinct from those activated by CGRP, cannot be excluded.

In conclusion, the present findings provide additional evidence that CGRP receptors are heterogeneous: the $CGRP_1$ /$CGRP_2$ receptor classification may need adaptation to accommodate the findings obtained with βCGRP and amylin as agonists at the putative CGRP receptor subtypes. Amylin acts as an agonist at the CGRP receptor in the guinea-pig atrium and may have preferential affinity for $CGRP_2$ vs. $CGRP_1$ receptors.

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