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Postjunctional inhibition of contractor responses in the mouse vas deferens by rat and human calcitonin gene-related peptides (CGRP)

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1 The effects of rat and human α-calcitonin gene-related peptide (CGRP) were compared in the mouse and rabbit isolated vas deferens preparation contracted by either field stimulation or acetylcholine.

2 The peptides were about equipotent at inhibiting twitch responses of the mouse vas deferens to field stimulation at 0.2 Hz (IC_{50} 12 ± 4 nM and 15 ± 3 nM, rat and human α-CGRP respectively). Rat α-CGRP was less potent at inhibiting responses to 10 Hz than to either 0.2 Hz or 1.0 Hz stimulation. The potency of rat α-CGRP at 1.0 Hz was unaltered by halving the calcium concentration of the Krebs solution.

3 The inhibitory effect of human α-CGRP was not antagonized by either propranolol (300 nM) or idazoxan (300 nM), although in the same tissues these latter two drugs reduced responses to isoprenaline and clonidine respectively.

4 Rat α-CGRP (100 nM) and human α-CGRP (1.0 μM) did not alter the uptake of [^{3}H]-noradrenaline (30 nM) into mice isolated vasa deferentia. Rat α-CGRP (3–100 nM) did not alter the fractional release per pulse (1.0 Hz, 100 pulses) of tritium from vasa preloaded with [^{3}H]-noradrenaline, although at the same time the peptide inhibited responses of the smooth muscle to field stimulation.

5 Rat and human α-CGRP were equipotent at inhibiting contractions of the mouse vas deferens evoked by acetylcholine although the peptides were less potent than against twitch responses.

6 In the rabbit vas deferens neither rat nor human α-CGRP (3 nM–1 μM) inhibited either twitch responses or acetylcholine contractions.

7 These results suggest that rat and human α-CGRP inhibit contractor responses of the mouse vas deferens not by interference with adrenergic mechanisms, but through postjunctional (possibly CGRP) receptors. A similar mechanism may underlie effects of CGRP in other tissues. The rabbit vas deferens appears to lack the CGRP 'receptors'.

Introduction

The calcitonin gene-related peptides (CGRP) are a new family of neuropeptides and contain 37 amino acids. The first to be identified was rat α-CGRP, an alternative gene product of the calcitonin gene (Rosenfeld *et al.*, 1983). Using immunocytochemical techniques these authors reported the presence of the peptide in a variety of tissues, including the brain, the heart and the vasculature. Subsequently a second CGRP gene has been identified in the rat and this encodes β-CGRP which differs from α-CGRP by a single amino acid (Rosenfeld *et al.*, 1984).

Analysis of the human calcitonin gene led to the identification of human α-CGRP (Nelkin *et al.*, 1984; Steenbergh *et al.*, 1984; Craig *et al.*, 1985; Edbrooke *et al.*, 1985). This peptide has been purified from medullary thyroid carcinoma tissue (Morris *et al.*, 1984). Human CGRP and its binding sites are widely distributed in human brain and spinal cord (Tschopp *et al.*, 1985). A second human CGRP gene encoding a peptide which differs by 3 amino acids from human α-

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CGRP has recently been identified (Steenbergh *et al.*, 1985).

A number of biological effects of these peptides have already been described. These include inhibition of gastric acid secretion (Tache *et al.*, 1984; Hughes *et al.*, 1984) and antinociception (Bates *et al.*, 1984). Given intracerebroventricularly, rat α-CGRP increased the blood pressure, heart rate and plasma noradrenaline levels in rats (Fisher *et al.*, 1983). These latter results suggested that the peptide acted centrally to increase sympathetic tone. However, the extent to which any effects of CGRP are dependent on sympathetic neurones remains to be established

One tissue which has been widely used in studies of sympathetic neurotransmission is the mouse isolated vas deferens. This preparation contains a high concentration of noradrenaline (Sjöstrand, 1965) and the amine can be released from the neurones by stimulation (Farnebo & Malmfors, 1971). There are a large number of different receptors in the vas deferens including those for various peptides, e.g. enkephalins (Hughes *et al.*, 1975), substance P (von Euler & Hedquist, 1974) and neuropeptide Y and peptide YY (Allen *et al.*, 1982).

Rat and human α-CGRP have a positive chronotropic effect in the rat isolated perfused heart but not in the heart of the rabbit (Marshall *et al.*, unpublished observations). Therefore, in the present experiments we have compared the effects of the peptides in the mouse and rabbit vas deferens. The results show that CGRP probably acts postjunctionally in the mouse vas deferens (independently of adrenergic mechanisms) to inhibit contraction. The CGRP 'receptors' are not present in the rabbit vas deferens.

Methods

Mouse isolated vas deferens

Male T.O. strain mice (30–40 g) were killed by stunning and cervical dislocation. The vasa deferentia were dissected out and placed under 0.5 g tension in a modified Krebs solution (composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11), maintained at 37°C and bubbled with 95% O_2 and 5% CO_2. The tissue was electrically field stimulated using two parallel platinum or silver electrodes and a Grass S44 or S48 stimulator at frequencies of 0.2, 1.0 or 10 Hz, 2.0 ms pulse width and 64 V. Contractions of the vas deferens were recorded isometrically via a Grass FT.03 transducer attached to a Grass polygraph.

Cumulative drug concentration-effect curves were obtained against 0.2 Hz and 1.0 Hz stimulation. The effect was determined by measuring the mean of 3 twitches when the response to the drug had stabilized and comparing this with the mean of the preceding 3 control twitches. IC_{50} values, the molar concentration of drug inhibiting the twitch by 50%, were calculated for individual experiments by linear regression analysis using log concentration and inhibitions between 20% and 80%. After a cumulative curve the tissue was washed and stimulated at 20 min intervals until either the original twitch height had returned or until two successive periods of stimulation gave consistent responses. The effect of drugs in the absence and presence of propranolol was assessed in separate groups of experiments. Idazoxan was added to the Krebs solution after control responses to drugs had been obtained and allowed to equilibrate for at least 10 min.

In experiments using 10 Hz stimulation, a single dose of drug was added to the tissue bath 30 s before the onset of a train of 50 pulses, sufficient to allow the response of the vas deferens to reach a maximum. The effect of CGRP was determined by comparing the maximum tension developed during stimulation in the presence of peptide with the mean maximum tension of the two preceding control responses to stimulation, each one separated by at least 10 min. After washing out the drug, control responses to stimulation were repeated until they were stable before a further dose of drug was added.

In some experiments acetylcholine, 30 μM, was added at 8 min intervals to elicit a contraction. After 15 s, by which time the peak response had occurred, the acetylcholine was washed out. CGRP was added 1 min prior to acetylcholine. The effect of CGRP was calculated by comparing the maximum tension developed by the vas deferens to acetylcholine in the presence and absence of the peptide. The mean of the 2 control responses to acetylcholine preceding the addition of CGRP was used for this calculation. Only after the restoration of consistent responses to acetylcholine was another dose of CGRP given. The doses of peptide were given in a random order in each experiment.

[^{3}H]-noradrenaline uptake and release

Vasa deferentia were removed from mice (as above) and incubated with 1-[7,8-^{3}H]-noradrenaline, 30 nM, for 10 min at 37°C in the presence or absence of either rat α-CGRP, 100 nM, or human α-CGRP, 1.0 μM. After washing, vasa were blotted dry, weighed and homogenised with a Polytron. The sample was suspended in Insta-Gel (1:1 ratio) and counted for tritium in a liquid scintillation spectrometer (Packard Tricarb 4660). Efficiency of counting was determined by an internal quench curve.

To study the release of tritium by electrical stimulation, vasa were loaded with 1-[7,8-^{3}H]-noradrenaline, 590 nM, for 30 min and set up as previously described (Marshall, 1983). Stimulation at 1.0 Hz, 100 pulses,

2 ms pulse width was via 2 parallel platinum/iridium electrodes using a Grass S48 stimulator. Successive stimulations were separated by at least 20 min. Contractions were recorded isometrically with a Grass FT.03 transducer attached to a Grass polygraph. CGRP was added 30 s prior to stimulation using a micrometer syringe. The Krebs solution in contact with the vas for 140 s was collected as the pre-stimulation sample. In the following 140 s period, stimulation began after 30 s and the bath was emptied 10 s after the train of pulses (see Marshall, 1983). The tritium in the Krebs solution was suspended in Insta-Gel and counted as described above. The results have been expressed as the fractional release of tritium (stimulation overflow of tritium minus prestimulation tritium overflow expressed as a fraction of the total tritium in the tissue at the time of stimulation) per pulse of electrical stimulation.

Rabbit isolated vas deferens

New Zealand white rabbits (3.4–4.1 kg) were killed and their vasa deferentia removed. The 2 cm of vas deferens nearest to the prostate was removed (the prostatic portion) and a similar length from the end adjacent to the epididymis (the epididymal portion).

Treatment of results

All results are given as mean ± s.e.mean. Differences between treatments have been compared using Student's t test for paired or unpaired observations. When $P < 0.05$, values were considered to be statistically significant.

Drugs

Rat α-CGRP and human α-CGRP were obtained from Peninsula Laboratories and subjected to mass spectrometry (M-Scan Ltd) and high performance liquid chromatography to confirm structure and purity before use. Stock solutions of the peptides (1.0 mM) were made in distilled water and have been kept at −20°C for up to 2 months without noticeable change in biological activity. Appropriate dilutions with Krebs solution were made prior to use, usually fresh daily.

Other drugs used were acetylcholine chloride (Sigma), isoprenaline sulphate (Blenkinsop), clonidine hydrochloride (Boehringer Ingelheim), propranolol hydrochloride (Sigma), idazoxan (Reckitt & Colman), disodium EDTA (BDH), (−)-ascorbic acid (Sigma), 17-β-oestradiol (Sigma). 1-[7,8-^{3}H]-noradrenaline hydrochloride (specific activity 35–40 Ci mmol^{-1}, or 1.3–1.5 TBq mmol^{-1}, Radiochemical Centre, Amersham) was diluted with deoxygenated distilled water to 5.9 μM and stored at 4°C.

Results

The response of the mouse vas deferens to stimulation

The resting tension of the mouse vas deferens was unaltered by the addition of either rat α-CGRP or human α-CGRP up to 300 nM.

The twitch response of the vas to 0.2 Hz stimulation was inhibited by rat and human α-CGRP. The effect of a given dose reached a maximum within about 45 s. In preliminary experiments the cumulative administration of CGRP gave reproduceable concentration-effect curves. Rat and human α-CGRP produced concentration-dependent inhibition and were approximately equipotent with IC_{50}s of 12.0 ± 4.1 nM and 14.5 ± 2.5 nM respectively. The maximum twitch inhibition elicited by the peptides was in excess of 80% (Figure 1). After washing the tissue, recovery from these cumulative curves was usually complete within 40 min.

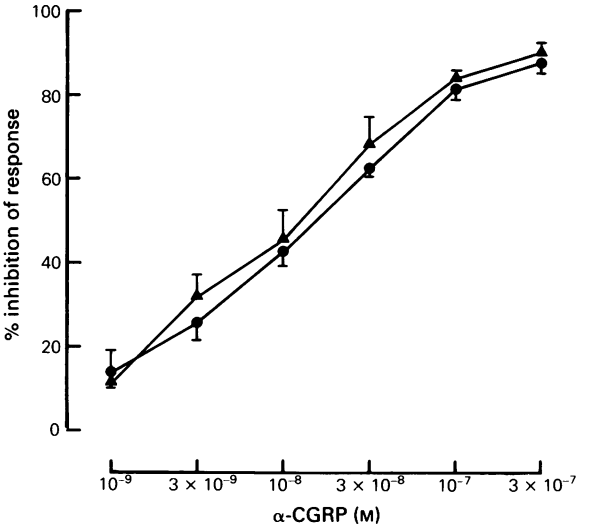

Figure 1 The percentage inhibition of twitch responses (0.2 Hz, 2 ms) of the mouse vas deferens by rat (▲) and human (●) α-calcitonin gene-related peptide (CGRP). Points represent means from at least 4 experiments and vertical lines are s.e.mean.

The inhibitory effect of rat α-CGRP was studied in responses evoked by different frequencies of stimulation. Control responses to stimulation at 0.2 Hz produced a tension of 177 ± 42 mg, while at 1.0 Hz and at 10 Hz it was 203 ± 12.5 mg and 1.56 ± 0.07 g respectively. The rat peptide was equi-effective at 0.2 and 1.0 Hz (IC_{50} 12.0 ± 4.1 nM and 13.4 ± 4.4 nM respectively) but less potent against the larger contraction in response to 10 Hz (IC_{50} 61 ± 13.6 nM, Figure 2).

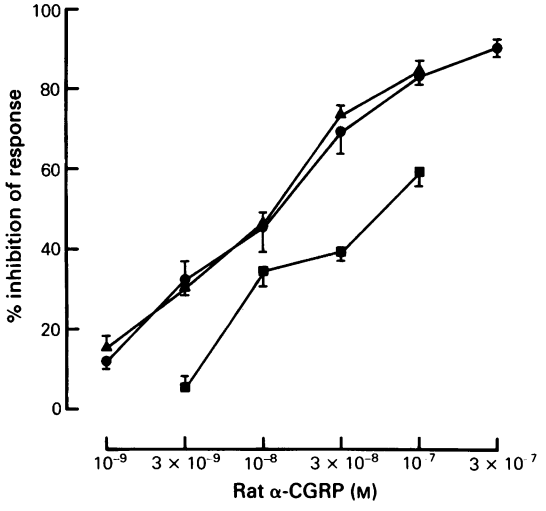

Figure 2 The percentage inhibition of the maximum tension developed to 0.2 Hz (●), 1.0 Hz (▲) or 10 Hz (■) stimulation of the mouse vas deferens by rat α-CGRP. Points represent means from at least 4 experiments and vertical lines are s.e.mean.

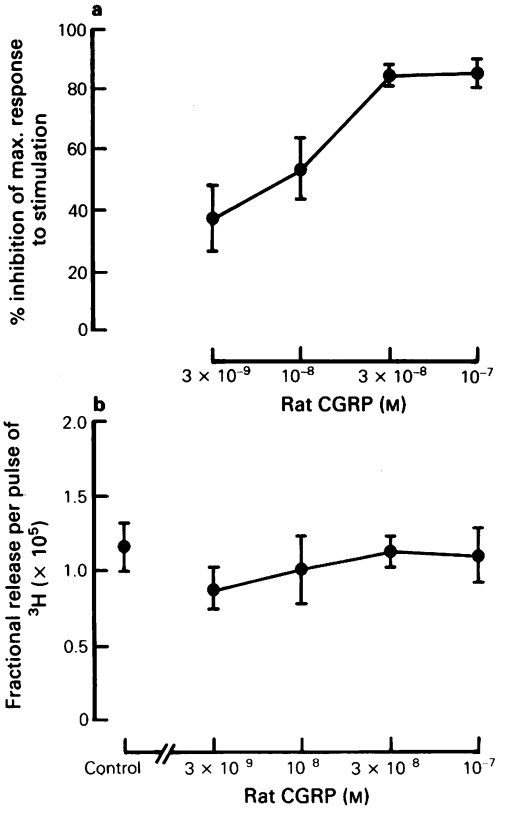

Figure 3 The effect of rat α-CGRP on the maximum tension (a) developed to 1.0 Hz, 100 pulses and on the fractional release per pulse of tritium (b) from mice vasa deferentia preloaded with [^{3}H]-noradrenaline. Points represent means from 4 experiments and vertical lines are s.e.mean.

In some experiments at 1.0 Hz stimulation the calcium content of the modified Krebs solution was reduced from 2.5 to 1.25 mM. This reduced the control response to stimulation at 1.0 Hz to 72.5 ± 21.0 mg. Rat α-CGRP (1, 3, 10, 30 and 100 nM) was equally effective in inhibiting responses either in the higher or lower calcium concentration (IC_{50} 9.6 ± 1.1 nM and 8.0 ± 0.3 nM respectively). The highest concentration of peptide, 100 nM, inhibited the response to stimulation by 85 ± 2% and 88 ± 1% in 2.5 and 1.25 mM calcium respectively.

Potential mechanisms underlying the CGRP twitch inhibition were investigated using adrenoceptor antagonists. Human α-CGRP was more than 30 times more potent than isoprenaline at inhibiting twitch responses (0.2 Hz) of the vas deferens. Propranolol, 300 nM, produced about a 10 fold shift to the right of the isoprenaline twitch-inhibition curve (IC_{50} 0.9 ± 0.2 and 7.0 ± 2.0 μM) but, in the same tissues, did not alter the inhibitory potency of human α-CGRP. In other experiments the α_2-adrenoceptor antagonist idazoxan, 300 nM, did not reduce the inhibitory effect of human α-CGRP (1–300 nM). However, idazoxan in the same tissues significantly antagonized the effect of the α_2-adrenoceptor agonist clonidine 3 nM (from 29 ± 6% inhibition to 7 ± 2%).

The uptake and relase of [^{3}H]-noradrenaline

The possibility of presynaptic inhibition of the neuronal uptake of [^{3}H]-noradrenaline by CGRP was investigated. In control vasa the uptake of [^{3}H]-noradrenaline, 30 nM, was 5.33 ± 0.34 nCi (197.1 ± 12.5 Bq) per mg weight of vas deferens. The presence of rat α-CGRP, 100 nM (a concentration which inhibited twitch responses by up to 80%), had no effect on the accumulation of tritium by vasa (5.46 ± 0.60 nCi or 202 ± 22 Bq per mg weight, *t* test $P > 0.05$). The even higher concentration of human α-CGRP, 1.0 μM, was similarly ineffective at reducing tritium uptake ($P > 0.05$).

In a further attempt to find a presynaptic action underlying the twitch inhibition by CGRP, the release of [^{3}H]-noradrenaline was studied. The control fractional release of tritium per pulse by a train of 100 pulses at 1.0 Hz was 1.17 ± 0.12 × 10^{-5}. The release of tritium was associated with contractions of the vas deferens leading to a maximum tension of 420 ± 30 mg. The addition of rat α-CGRP (3–100 nM)

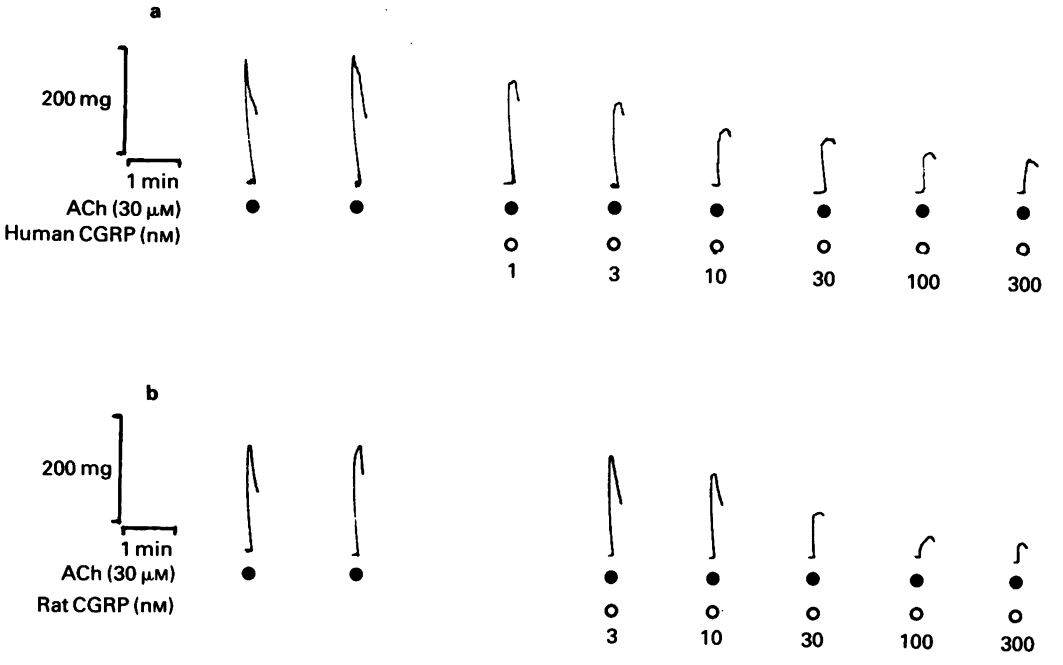

Figure 4 Examples of the inhibition of contractions to acetylcholine (ACh), 30 μM, in the mouse vas deferens by human α-CGRP (a) and rat α-CGRP (b).

did not alter the basal release of tritium or the resting tension of the tissue. However, the peptide produced a concentration-dependent inhibition of the tension response without affecting the fractional release per pulse of tritium (Figure 3).

Acetylcholine contractions of the vas deferens

To investigate a possible postjunctional site of action for CGRP the mouse isolated vas deferens was contracted by acetylcholine. Preliminary experiments showed that acetylcholine (1.0–300 μM) elicited concentration-dependent contractions which were antagonized by atropine, 10 nM. A concentration of acetylcholine, 30 μM, which produced responses about 60% of the maximum, was used in the present studies and gave a contraction of 284 ± 19 mg tension. Rat and human α-CGRP, added 1 min before acetylcholine, inhibited contractions of the vas deferens (Figure 4). Both peptides produced concentration-dependent inhibition and were approximately equipotent (Figure 5). Comparison of these results with those of twitch inhibition by the two CGRPs (Figure 1) show that the peptides were less potent at inhibiting contractions evoked by acetylcholine.

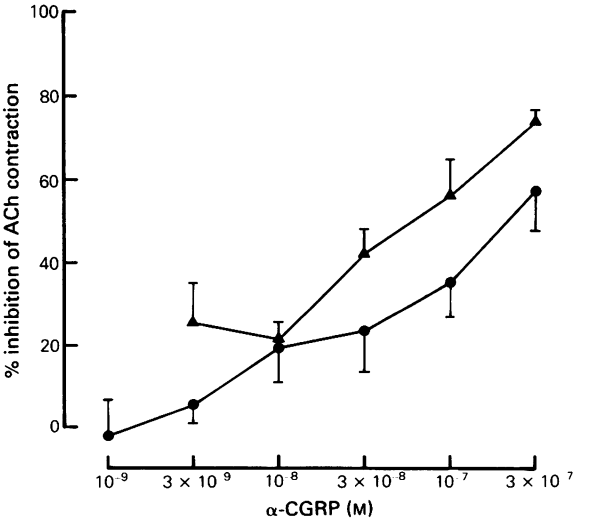

Figure 5 The percentage inhibition of contractions to acetylcholine (ACh), 30 μM, in the mouse vas deferens by rat (▲) and human (●) α-CGRP. Points represent means from at least 4 experiments and vertical lines are s.e.mean.

Rabbit vas deferens

In vasa from seven rabbits, twitch responses to stimulation at 0.2 Hz of both the prostatic and epididymal portions of the rabbit vas deferens (952 ± 221 and 484 ± 70 mg respectively) were not significantly altered by the cumulative addition of either rat or human α-CGRP (3.0 nM–1.0 μM). Contractions of both portions of the vasa to acetylcholine, 30 μM, also were unaffected by rat or human α-CGRP, 100 nM and 1.0 μM.

Discussion

The twitch response of the mouse isolated vas deferens to field stimulation can be inhibited by rat and human α-CGRP. This finding raises the possibility that these neuropeptides may exert their biological effects by affecting neurotransmission. In this context the mechanism of action underlying the twitch inhibition may be of great importance.

The present work has centred on adrenergic mechanisms because rat α-CGRP may increase central sympathetic outflow (Fisher *et al.*, 1983) and it was of interest to see if the peptide directly affected peripheral adrenergic neurones or adrenoceptors. Therefore, the mouse vas deferens has been used as a model system as the twitch response can be inhibited in a variety of ways by drugs influencing adrenergic mechanisms. For example, responses of the mouse vas deferens to field stimulation can be inhibited by indirect acting sympathomimetics (Marshall *et al.*, 1978b), neuronal uptake blocking drugs (Jenkins *et al.*, 1977), α_2- or β-adrenoceptor agonists (Marshall *et al.*, 1978a; Jenkins *et al.*, 1977) and adrenergic neurone blocking agents (Marshall *et al.*, 1980). All these possible mechanisms of action for CGRP have been eliminated as the peptide did not alter either the uptake of [^{3}H]-noradrenaline, or its basal or stimulated release and the twitch inhibition was not antagonized by the α_2-adrenoceptor antagonist idazoxan or by propranolol.

Twitch responses of the mouse vas deferens can also be inhibited via opioid receptors (Hughes *et al.*, 1975) and histamine H_2-receptors (Marshall, 1981). Rat α-CGRP was equi-effective against responses to stimulation at 0.2 Hz and 1.0 Hz but less effective against responses of the vas deferens elicited by 10 Hz which is similar to histamine (Marshall, 1981), but different from α_2-adrenoceptor or opioid agonists (Marshall *et al.*, 1978a; 1981). An additional characteristic of twitch inhibition of rat α-CGRP was its lack of dependence on the external calcium concentration. This differentiates its action from that of α_2-agonists (Marshall *et al.*, 1980), opioids (Marshall *et al.*, 1981) as well as from histamine (Marshall, 1981) where decreasing the calcium concentration leads to an increased effectiveness of the drug.

In the mouse vas deferens, clonidine and morphine inhibit the release of [^{3}H]-noradrenaline (Marshall, 1983; Hughes *et al.*, 1975) in addition to inhibiting twitch responses. This presynaptic effect is shared by neuropeptide Y which inhibited responses to field stimulation and also decreased the evoked efflux of noradrenaline in the rat vas deferens (Lundberg & Stjarne, 1984). Unlike neuropeptide Y, CGRP, in concentrations markedly inhibiting the smooth muscle responses to stimulation, did not alter either the uptake or the release of the amine. Therefore the present experiments provide no evidence in support of a presynaptic action for CGRP in the mouse vas deferens.

An alternative (or additional) site of action for CGRP is a postjunctional one. To investigate this possibility vasa were contracted using acetylcholine. Rat and human α-CGRP were about equipotent at inhibiting these contractions. The finding that the peptides were less potent against acetylcholine compared with field stimulation may reflect differences in receptor linked contractor mechanisms rather than suggest that the two inhibitory effects are differently mediated. The possibility that CGRP blocked muscarinic receptors is unlikely as rat and human α-CGRP (up to 1.0 μM) did not alter contractions to acetylcholine (30 μM) in the rabbit vas deferens. The ability of CGRP to inhibit contractions evoked by an exogenous agonist in the mouse vas deferens contrasts with the finding that neuropeptide Y did not alter noradrenaline contractions in the mouse (Allen *et al.*, 1982) or rat vas deferens (Lundberg *et al.*, 1982; Ohhashi & Jacobowitz, 1983).

The simplest explanation for all the results from the present experiments is that CGRP acts postjunctionally to inhibit contractions of the vas deferens evoked either by released transmitter or by acetylcholine. In the absence of any evidence that the peptides act directly (or indirectly) through a known receptor, it is possible that CGRP acts through its own receptors which may be sited on the smooth muscle. While there is no other evidence for these receptors in the vas deferens, binding sites for CGRP have been identified e.g. in the human central nervous system, pituitary and spinal cord (Tschopp *et al.*, 1985).

The lack of effect of CGRP in the rabbit vas deferens contrasts with its inhibitory effects in the mouse tissue. A similar species difference has been observed with other peptides in the vas deferens. For example, twitch responses of the mouse vas deferens can be inhibited by the opioid δ-receptor agonist [D-Ala^2, D-Leu^5] enkephalin and by the μ-receptor agonist [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin (Gillan *et al.*, 1981) but the same compounds are inactive against the twitch response of the rabbit vas deferens (McKnight *et al.*, 1982). Therefore, in the present experiments, the

lack of effect of rat and human α-CGRP in the rabbit vas deferens may suggest either that there are no CGRP 'receptors' present or that, if present, they differ from those in the mouse vas deferens.

In conclusion, contraction of the mouse vas deferens by either nerve stimulation or by exogenous agonist can be inhibited by the neuropeptides rat and human α-CGRP. Therefore, this effect is probably mediated postjunctionally and possibly through a CGRP 'receptor' (which may be absent from the rabbit vas deferens). Such a mechanism may have wider application than the vas deferens and potentially provides a way in which the calcitonin gene-related peptides could modulate the actions of neurotransmitters and hormones elsewhere in the body.

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