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Calcitonin gene-related peptide (CGRP) is a potent non-endothelium-dependent inhibitor of coronary vasomotor tone

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1 Ring segments of bovine left circumflex coronary artery were pre-contracted with 5-hydroxytryptamine or phenylephrine and then exposed to increasing concentrations of calcitonin gene-related peptide (CGRP) and other drugs.

2 CGRP administration resulted in dose-dependent inhibition of induced tone. Maximal relaxation to CGRP was 89 ± 5% and the concentration required to achieve 50% maximal relaxation (EC_{50}) was $2.11 \pm 1.35 \times 10^{-9}$ M.

3 CGRP-induced relaxation was not affected by removal of endothelial cells nor was it significantly altered by incubation of coronary vessels with atropine, propranolol, phentolamine (all 10^{-6} M) or indomethacin (10^{-5} M).

4 From these data we conclude that CGRP is a potent inhibitor of coronary artery vasomotor tone which appears to act directly on vascular smooth muscle rather than through the release of a secondary mediator. These data support the possibility that CGRP may play a role in non-adrenergic, non-cholinergic regulation of coronary artery tone.

Introduction

Calcitonin gene-related peptide (CGRP), a 37 amino acid peptide, is the major product of the calcitonin gene in neural tissue (Rosenfeld *et al.*, 1983). CGRP is widely distributed throughout the central and peripheral nervous systems in mammals (Springall *et al.*, 1983; Rosenfeld *et al.*, 1983; Terenghi *et al.*, 1985) and there is evidence that CGRP in plasma is derived mainly from perivascular nerves (Zaidi *et al.*, 1985). CGRP immunoreactive nerve fibres have been identified in high concentration in cardiac tissue (Saito *et al.*, 1986) and they appear to be particularly abundant in the walls of coronary blood vessels (Lundberg *et al.*, 1985; Mulderry *et al.*, 1985). Although CGRP is a potent inhibitor of vasomotor tone in systemic vascular beds (Brain *et al.*, 1985; Hanko *et al.*, 1985), its effects on coronary arteries are uncertain. In addition, several studies have presented evidence suggesting that the vasodilator effects of CGRP are indirect and may involve the release of secondary mediators such as the endothelium-derived relaxant factor (EDRF) (Brain *et al.*, 1985), prostaglandins (Brain *et al.*, 1985) or even catecholamines (Fischer *et al.*, 1983; Etienne *et al.*, 1984). The present study was performed to assess the effects of CGRP on bovine coronary artery (CA) tone and to determine whether other mediator substances were involved in this process.

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Methods

Tissue preparation

Hearts were obtained at a local abbatoir where young cattle were killed and immediately exsanguinated. The proximal segment of the left circumflex CA was dissected free of epicardial fat and connective tissue. Vessels were placed in ice cold Krebs-Henseleit (KH) solution which had been pregassed with 95% O_2:5% CO_2 for transport to the laboratory. The KH solution had the following composition (mM): NaCl 118, KCl 5.9, $MgSO_4.7H_2O$ 2.5, NaH_2

$PO_4.H_2O$1.2, $CaCl_2.6H_2O$2.5, glucose 5.6 and Na HCO_3 25.5. Ring segments 2–3 mm in width were sectioned from the centre of each vessel. In half of these, endothelial cells were removed by gently rotating a steel probe through the luminal surface for 30 s. Following completion of functional studies, the presence of endothelial cells was confirmed by histological evaluation in a random sample of 25% of the segments using a modification (Griffith *et al.*, 1984) of the silver staining technique described by Poole *et al.* (1958). Unrubbed segments had endothelial cells over 60–80% of the luminal surface whereas the rubbed segments were virtually denuded of endothelial cells. Ring segments were suspended by means of gently curved stainless steel hooks in 10 ml glass chambers filled with KH solution which was aerated with 95% O_2:5% CO_2. The pH of the buffer solution was 7.4. The lower hook was attached to a fixed support and the upper was tied with 3–0 silk thread to a Grass FT.03 force transducer which was mounted on a micrometer apparatus. Changes in isometric force were recorded on a Grass 7D polygraph. The chambers were maintained at 37°C by immersing them in an outer water bath which was warmed by a recirculating heater. A force of 6 g was applied to the vessels. This level of preload was determined to be optimal for maximizing the response to a constricting agent in bovine coronary arteries of the size used in these experiments. This force was maintained as necessary and the KH replaced every 15 min during a 2 h equilibration period. At the conclusion of this period, tissue viability and the magnitude of the contractile response was assessed by increasing the potassium concentration of the KH to 135 mM.

Pharmacological studies

Since bovine CA segments have little or no intrinsic tone, relaxation could not be evaluated in the basal state. Consequently, the vessels were pre-contracted to 50% of the level observed during exposure to high potassium solution by adding either 5-hydroxytryptamine (5-HT) or phenylephrine (PE) so that the final concentration of these agents in the bath ranged from 80–500 nM and 1–10 μM, respectively. Functional assessment of the presence of endothelial cells was performed by adding increasing concentrations of either acetylcholine (ACh) or adenosine triphosphate (ATP) to the pre-contracted vessels. The response to increasing concentrations of CGRP was tested at baseline and after pre-incubation of the CA segments for 30 min with either 1 μM each of atropine, propranolol and phentolamine ($n = 11$) or 10 μM of indomethacin ($n = 10$). Relaxation was also tested by exposing pre-contracted vessel segments to increasing concentrations of vasoactive intestinal peptide (VIP), and isoprenaline.

Drugs

ACh, ATP, isoprenaline, propranolol, atropine, 5-HT, PE (all from Sigma, Poole, U.K.) and phentolamine mesylate (supplied as a gift from Ciba Pharmaceutical, U.K.) were prepared in distilled water. Indomethacin (Sigma) was dissolved in 100% ethanol and further diluted with distilled water. These drugs were freshly prepared on the day of study. CGRP and VIP (Bachem, Torrance, Ca., U.S.A.) were dissolved in 0.1 N acetic acid containing bovine serum albumin (200 μg mg^{-1} peptide). Aliquots of a concentrated solution of these were then frozen at −20°C and used within 60 days of preparation.

Data analysis

Cumulative relaxation to increasing concentrations of drug were measured with each concentration remaining in contact with the tissue until the response at that level was complete. Maximal relaxation is defined as the greatest relaxation of induced tone possible with a drug compared to relaxation seen following exposure to 100 μM of papaverine and is expressed as a percentage. The EC_{50} is the concentration of drug required to achieve 50% maximal relaxation and is expressed as its negative logarithm. The EC_{50} was determined from the regression equation describing the relationship between drug concentration and relaxation with the latter expressed as its probit transformation. Data are given as mean ± standard error (s.e.). Results were evaluated using paired and unpaired t tests where appropriate. Differences with a P value less than 0.05 were considered significant.

Results

The response to CGRP was assessed in 28 bovine coronary artery segments half of which had been rubbed to remove endothelial cells (Table 1). The presence or absence of endothelial cells was confirmed in each preparation by assessing the response to either ACh or ATP. Levels of pre-contraction did not differ significantly between vessels exposed to 5-HT and those exposed to PE. However, contraction was significantly greater in rubbed than in unrubbed segments despite the use of identical amounts of 5-HT or PE. As shown in Figure 1, the response to CGRP in segments with and without endothelial cells was similar. The results from studies performed in 14 pairs of vessels are summarized in Table 1. Relaxation was similar in vessels contracted with 5-HT and PE. The dose-response curve for relaxation in all segments exposed to CGRP is shown in Figure 2. In order to determine whether relaxation depended on the release of a secondary mediator, the response to CGRP before

Table 1 Relaxation of bovine coronary artery induced by various agents

Agent	n	*EC*	*Level of contraction (g)*	*Maximal relaxation (%)*	EC_{50} *(M)*
CGRP	14	−	8.2 ± 0.8*	85 ± 6	8.59 ± 0.13
	14	+	5.9 ± 0.7	93 ± 4	8.76 ± 0.13
VIP	3	−	7.9 ± 1.5	83 ± 7	8.53 ± 0.06
	3	+	4.1 ± 0.7	83 ± 8	8.50 ± 0.11
ACh	5	−	5.2 ± 1.4	0*	
	11	+	5.3 ± 0.9	84 ± 4	7.43 ± 0.08
ATP	6	−	8.0 ± 1.6*	61 ± 9**	
	6	+	3.9 ± 0.3	99 ± 1	5.32 ± 0.11
Iso	5	−	9.4 ± 1.8*	98 ± 2	7.68 ± 0.13
	6	+	4.3 ± 0.5	99 ± 1	8.02 ± 0.10

Abbreviations: EC = endothelial cells; + = present; (−) = absent; ATP = adenosine triphosphate; ACh = acetylcholine; CGRP = calcitonin gene-related peptide; VIP = vasoactive intestinal peptide; Iso = isoprenaline.
*$P < 0.05$ when rubbed segments are compared to unrubbed segments; ** represents relaxation observed at 10^{-3} M ATP which was the highest concentration used in these experiments.

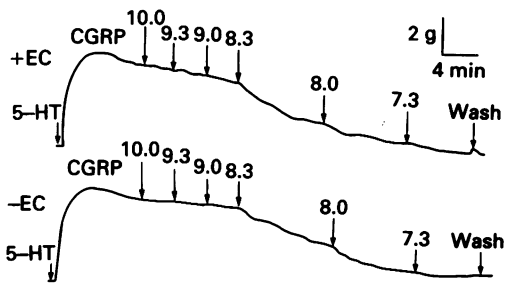

Figure 1 Effects of calcitonin gene-related peptide (CGRP) on bovine coronary artery segments. In a previous experiment, the top segment, which had endothelial cells (EC) present, demonstrated nearly complete relaxation to acetylcholine while the bottom segment, which had been denuded of EC, showed no evidence of relaxation. In this experiment, both segments were pre-contracted with 5-hydroxytryptamine (5-HT). Increasing concentrations of CGRP (expressed as its negative logarithm) in the bath resulted in nearly identical relaxation of the 2 segments.

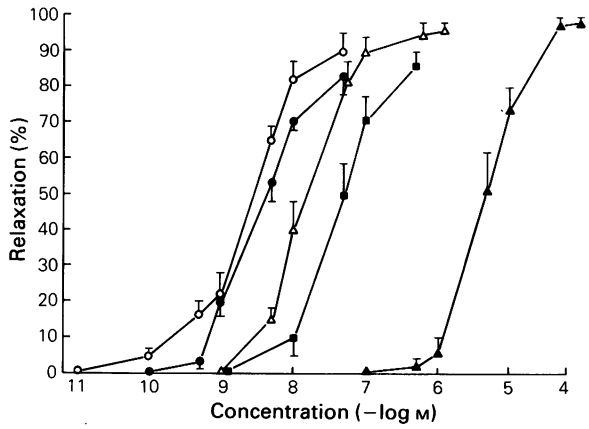

Figure 2 Dose-response curves for relaxation of bovine coronary artery. The effects of calcitonin gene-related peptide (○, $n = 28$), vasoactive intestinal peptide (●, $n = 6$), isoprenaline (△, $n = 11$), acetylcholine (■, $n = 11$), and adenosine triphosphate (▲, $n = 6$) are shown. Only segments with endothelial cells were used to assess the response to acetylcholine and adenosine triphosphate. Points are mean values with s.e.mean shown by vertical lines.

and following 30 min incubation with atropine, phentolamine, and propranolol (all 1 μM) or following indomethacin (10 μM) was compared. As shown in Table 2, these drugs did not significantly alter relaxation to CGRP.

The effects of VIP, isoprenaline, ACh and ATP on pre-contracted bovine CA segments are summarized in Table 1. VIP and isoprenaline were similar to CGRP in that relaxation was not affected by removing endothelial cells. In contrast, ACh relaxed bovine CA segments only when endothelial cells were present. The response to ATP, while strongly influenced by the presence of endothelial cells, was not entirely endothelium-dependent. At high concentrations of ATP, relaxation was seen in rubbed segments. At a concentration of 10^{-3} M ATP, relaxation averaged 61 ± 9%. Higher concentrations of ATP were not used so that

Table 2 Effects of blocking agents on calcitonin gene-related peptide (CGRP)-induced relaxation

Condition	n	*Maximal relaxation* (%)	EC_{50} (M)
Control	11	92	8.50 ± 0.08
Adrenoceptor and cholinoceptor blockade	11	87	8.39 ± 0.08
Control	10	86	8.57 ± 0.05
Indomethacin	10	93	8.55 ± 0.08

neither maximal relaxation nor the EC_{50} concentration was calculated in the rubbed segments. The dose-response curves for these agents are shown in Figure 2. Although maximal relaxation was similar for all agents, the dose-response curve for CGRP was to the left of the other agents and comparison of EC_{50} values revealed that CGRP was approximately 10 fold more potent than either isoprenaline or ACh, and close to 100 fold more potent than ATP (Table 1).

Discussion

We have shown that CGRP is a potent inhibitor of bovine coronary artery tone. Although maximal relaxation was virtually complete for all the drugs tested, the EC_{50} concentration for CGRP was lower than for either isoprenaline, ACh or ATP. The inhibitory effects of CGRP on vasomotor tone have been shown in several mammalian vascular beds (Brain *et al.*, 1985; Lundberg *et al.*, 1985; Hanko *et al.*, 1985). However, evidence that this peptide acts directly on coronary vasomotor tone has not previously been presented. Marshall *et al.* (1986). using an isolated rabbit heart preparation, reported that CGRP infusion increased coronary flow and McEwan *et al.* (1985) demonstrated evidence of coronary vasodilatation during direct infusion of CGRP during coronary angiography in man. However, these studies did not differentiate direct vascular effects of CGRP from secondary effects due to the release of vasoactive substances that resulted from CGRP effects on myocardial tissue. Since isolated blood vessels were used in the present study, the influence of myocardial factors was avoided. Other workers have presented evidence in rat that CGRP effects are mediated at least in part by activation of adrenoceptors (Fischer *et al.*, 1983; Etienne *et al.*, 1984). Brain *et al.* (1984) presented evidence that CGRP-induced relaxation of rat aorta was both endothelium-dependent and that the effects were partially blocked by indomethacin. However, in our experiments CGRP effects were not altered by blockade of adrenoceptors or cholinoceptors, inhibition of prostaglandin production, or removal of the vascular endothelium. Although CGRP appears to act directly on bovine coronary smooth muscle tone the pathway through which relaxation occurred was not identified. Kubata *et al.* (1985) have presented evidence that cyclic AMP is involved in this process in rat aorta.

In view of the reportedly high concentration of CGRP-immunoreactive nerve fibres in the walls of coronary vessels from several species, (Lundberg *et al.*, 1985; Mulderry *et al.*, 1985) these data raise the possibility that CGRP may play a role in the regulation of coronary artery tone. There is evidence supporting the existence of non-adrenergic, non-cholinergic (NANC) neurogenic regulation of airway, (Matsuzaki *et al.*, 1980; Cameron *et al.*, 1983) genito-urinary (Snedden & Westfall, 1984) and cardiac smooth muscle (Saito *et al.*, 1986) and NANC relaxation of coronary (Rooke *et al.*, 1982) and other vessels (Duckles, 1979; Hamasaki *et al.*, 1983) has been demonstrated. However, the identity of the neurotransmitters in this third nervous system are still uncertain. There is evidence to suggest that neuropeptides may be involved in this process (Goyal *et al.*, 1980; Matsuzaki *et al.*, 1980; Cameron *et al.*, 1984; Goedert *et al.*, 1984) and our data support the possibility that CGRP may function as a neurotransmitter in the NANC regulation of coronary artery tone. However, until more is known about the generation and degradation of CGRP in the nervous system and a specific competitive antagonist becomes available, the physiological role of this peptide will remain speculative. An alternative possibility is that CGRP may alter vascular tone at a site that is distant from that where it is released. Zaidi *et al.* (1985) has shown that plasma CGRP arises from perivascular nerves and CGRP has been detected in plasma from patients with medullary thyroid carcinoma (Morris *et al.*, 1984) and from normal subjects (Girgis *et al.*, 1985). Our data show that bovine coronary arteries have receptors which can be activated by relatively low concentrations of CGRP.

In our experiments, ACh caused relaxation only in segments with endothelial cells. Similar findings have been reported by Holzmann (1982) in bovine coronary

arteries. The response to ATP in the present study was partially endothelial cell-dependent. Similar results with ATP have been noted previously in other blood vessels (DeMey *et al.*, 1982; Furchgott, 1983) and may be explained by the fact that high concentrations of ATP are broken down by the vessel wall to produce adenosine (Pearson & Gordon; 1979), an agent which causes non-endothelial-dependent relaxation. Release of endothelium-derived relaxing factor (EDRF) in response to ACh and other agents was first described by Furchgott & Zawadzki (1980) and now has been confirmed in numerous laboratories. Basal release of EDRF is the most likely reason for the significantly higher contractile response to 5-HT or PE that was seen in rubbed segments in most of the experiments. Interestingly, vasoactive intestinal peptide (VIP) effects on bovine coronary artery segments were not endothelial cell-dependent. Although endothelial cells are required for VIP to relax rat aorta (Davies & Williams, 1984), we have previously shown that relaxation of both bovine and human pulmonary arteries is not endothelial cell dependent (Greenberg *et al.*, 1987).

In conclusion, we have demonstrated that CGRP is a potent inhibitor of vasomotor tone in bovine coronary arteries and that it appears to act directly on vascular smooth muscle. These observations support the hypothesis that CGRP may play a role in regulating CA tone and blood flow.

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