Human calcitonin gene-related peptide activates adenylate cyclase and releases prostacyclin from human umbilical vein endothelial cells

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1 Endothelial cells of human umbilical vein were isolated and cultured in vitro.
2 In these cells there was a concentration-dependent release of prostacyclin and activation of adenylate cyclase by human calcitonin gene-related peptide (hCGRP). The concentration of hCGRP for half-maximum activation of adenylate cyclase (Km) by hCGRP was 190 nM.
3 Bradykinin induced a ten fold greater release of prostacyclin than CGRP, but did not activate adenylate cyclase.
4 hCGRP may exert its potent vasodilator properties by stimulating release of vasorelaxant substances, including prostacyclin from endothelial cells.

Introduction

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide which is produced by modification of the calcitonin gene product (Amara et al., 1982; Rosenfeld et al., 1983). Human CGRP (hCGRP) differs by 4 amino acids from the rat CGRP (Morris et al., 1984). In man it circulates in the blood at levels five times that of calcitonin (Girgis et al., 1985), and in rodents CGRP is widely distributed in the central nervous and cardiovascular systems (Skofitsch & Jacobowitz, 1985; Mulderry et al., 1985). CGRP is particularly abundant in perivascular nerves where it co-localizes with substance P (Lundberg et al., 1985). In some nerves it co-exists with acetylcholine (Takami et al., 1985). This suggests that CGRP may have a role in the control of vascular smooth muscle tone.

CGRP is a potent vasodilator in vitro, and has positive inotropic and chronotropic effects on the heart (Tippins et al., 1984; Brain et al., 1985). Systemic administration in both rodents and man causes vasodilatation with associated tachycardia (Fischer et al., 1983; Struthers et al., 1985). Marked dilatation of skin arterioles of rabbits and man is also seen following intradermal injection (Brain et al., 1985). Further, hCGRP is a potent dilator of human and porcine coronary vessels (McEwan et al., 1985; Greenwald et al., 1986).

It remains controversial whether the vasodilatation by CGRP in vitro is dependent on the presence of an intact endothelium. Endothelium-dependent relaxation has been found in a variety of human blood vessels and in rat aorta (Brain et al., 1985; Hughes et al., 1985) but relaxation is independent of endothelium in some rat, rabbit and cat vessels (Hanko et al., 1985; Edvinsson et al., 1985a, b). We have therefore examined the effect of hCGRP on endothelial cells of human umbilical vein. Measurements were made of hCGRP-dependent activation of adenylate cyclase (ATP pyrophosphate lyase (cyclizing): EC 4.6.1.1) and prostacyclin release.

Methods

Preparation of cells and cell culture

Human umbilical vein endothelial cells were prepared by a modification of a technique described previously (Jaffe et al., 1973). Umbilical cords were obtained from the obstetric units of Queen Charlotte’s and Hammersmith Hospitals (London) after 98 normal vaginal deliveries or elective Caesarian sections. Cords were selected from pregnancies not associated with foetal distress. The cords were transported in Dulbec-co’s phosphate buffered saline (PBS) (no calcium or magnesium ions). The clamp marks at either end of the

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cord were excised, and the ends of the umbilical vein were cannulated with two 3-way taps. The umbilical vein was then washed with PBS. The PBS was replaced by a solution of collagenase Type 2 (Sigma) 0.5 mg ml\(^{-1}\) in Dulbecco’s modified Eagle’s medium (DMEM) with 25 mM HEPES buffer, pH 7.4. The cord was then incubated for 20 min at 37°C. The collagenase solution was released from the cord into a plastic centrifuge tube containing 10 ml of DMEM and 20% foetal calf serum. After centrifugation at 150 g for 5 min, the pellet was resuspended, and cells grown in DMEM supplemented with 20% foetal calf serum, 1.5 mM glutamine, 50 mM EDTA and streptomycin (Gibco), 50 \(\mu\)g ml\(^{-1}\) gentamycin (Gibco) and 50 \(\mu\)l ml\(^{-1}\) mycostatin. Cells in each 25 cm\(^{2}\) flask were grown to confluence, and harvested in one of two ways. For the measurement of adenylate cyclase activity, cells were removed in PBS from the bottom of the flasks with a rubber policeman, and pelleted by centrifugation at 150 g for 5 min in plastic tubes. The pellets were stored at \(-80°C\), re-thawed and pooled. In experiments on the release of prostacyclin, cells were removed from the flask with 0.5 mg ml\(^{-1}\) trypsin (Sigma)/0.5 mM EDTA. The cells were pelleted as before and resuspended in culture medium before culture on microcarrier beads.

**Preparation of human endothelial cell membranes**

Cell homogenates were prepared by thawing the cell pellets and resuspending in 25 mM Tris-HCl buffer, pH 7.4, containing 0.32 mM sucrose (0.5 ml per flask of cells). Cells were disrupted with 30 strokes of a tightly fitting Dounce homogenizer, and intact cells and nuclei were removed by centrifugation at 300 g for 10 min at 4°C. Cell membranes were contained in a supernatant, which was diluted six fold with 50 mM Tris-HCl buffer, pH 7.4, containing 0.25 mM EDTA. The suspension was then centrifuged at 100,000 g for 20 min at 4°C. The pellet was suspended finally in 50 mM Tris-HCl buffer, pH 7.4 containing 0.25 mM EDTA and frozen at \(-80°C\) until required.

The membranes were thawed, homogenized with 10 strokes of a Dounce homogenizer, and maintained at 4°C in an ice bath immediately before use.

**Adenylate cyclase assay**

Enzyme activity was determined by a modification of the method of Salomon et al. (1974). The assay was carried out in triplicate. Each 100 \(\mu\)l reaction mixture contained 50 mM Tris-HCl buffer, pH 7.4; 5 mM magnesium chloride; 20 mM creatine phosphate, disodium salt (Sigma); 10 \(\mu\)l creatine kinase: 150 \(\mu\)g \(^{-1}\) protein (ATP: creatine N-phosphotransferase, EC 2.7.3.2) (Sigma); 1 mM cyclic adenosine 3', 5'-monophosphate (cyclic AMP), sodium salt (Sigma); 4 \(\mu\)M guanosine 5'-triphosphate (GTP), sodium salt (Sigma); 0.25 mM Ro20-1724 (a phosphodiesterase inhibitor, Roche Products Ltd); 0.5% ethanol; aprotinin 20 \(\mu\)l (Bayer); 1 mM [\(\alpha\)\(^32\)P]ATP (5 Ci, New England Nuclear; 29.58 Ci mmol\(^{-1}\): 1 Ci = 3.7 \times 10\(^{10}\) Bq); 0.1 mg ml\(^{-1}\) essentially fatty acid free bovine serum albumin (Sigma) and endothelial cell membranes (0.011–0.013 mg protein per tube). Reaction mixtures were incubated for 30 min at 37°C. For the time course reactions, single reaction tubes were set up containing appropriate volumes and reagents at the same molar concentrations. Volumes of 100 \(\mu\)l were removed at selected times, and all reactions terminated by the addition of 800 \(\mu\)l of 6.25% (w/v) trichloracetic acid. [\(\alpha\)\(^32\)P]-ATP and [\(\beta\)P]-cyclic AMP were separated by a two-step chromatographic procedure, and the losses of cyclic AMP were corrected for by measurement in each tube of the recovery of [\(\alpha\)H]-cyclic AMP (approx 10,000 c.p.m.; 23.6 Ci mmol\(^{-1}\), Amersham International). The production of [\(\beta\)P]-cyclic AMP increased linearly over 30 min in the absence of hCGP \((r = 0.98)\).

**Preparation of endothelial cells for perfusion studies**

The release of prostacyclin from endothelial cells was measured by the perfusion of these cells attached to microcarrier beads as described previously by Gordon & Martin (1983). Dispersed cells from four 25 cm\(^{2}\) flasks were added to 2.5 ml of Superbead TM microcarrier inert beads (Flow Laboratories) suspended in an intermittently stirring culture flask (Techne) containing 20 ml of the culture medium described above. The beads were stirred (3 min on, 30 min off) overnight. The number of cells per bead was counted after ethanol fixation and staining with 0.1% methyl violet. A column for perfusion was prepared from the barrel of a 2 ml syringe, plugged with siliconised glass wool. The suspension of beads was poured into the barrel, and the final packed volume was between 1.8 and 2 ml. A second siliconised glass wool plug was placed on the top of the column, and the syringe plunger, with a fine polythene delivery tube inserted through the centre, was then sited on top of the column. The column was perfused from the bottom upwards, at a rate of 0.35–0.375 ml min\(^{-1}\), with DMEM and 1 mg ml\(^{-1}\) essentially fatty acid-free bovine serum albumin, equilibrated with 95% air and 5% \(\mathrm{CO}_2\). Using dextran blue as a marker, it was demonstrated that at this perfusion rate the volume of the system was such that it took 4 min to perfuse it end to end. All studies were undertaken in a room maintained at 33–35°C. The column was perfused for 40 min to allow the system to return to a stable state. During an experiment, fractions of the perfusate were collected for periods of 1 min, and these were saved for assay of 6-oxo-PGF\(_{1\alpha}\). Cells were stimulated with
hCGRP for periods of 4 min. Although the amounts of hCGRP required for such perfusions are small, insufficient hCGRP was available for repeated experiments at 10 μM.

Measurement of prostacyclin

Prostacyclin was measured by radioimmunoassay of the stable hydrolysis product (6-oxo-PGF$_{1α}$). Samples of the perfusate to be assayed (50 μl) were added to the assay tubes without solvent extraction. The detection limit of the assay was <5 pg per sample, and there was no cross-reactivity with hCGRP. The antibody was a generous gift from Dr Myatt (Institute of Obstetrics, Royal Postgraduate Medical School).

The Ro20-1724 (4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone) was donated by Roche Products Ltd. The hCGRP was synthesized chemically (Bachem) and was the generous gift of Dr J. Pless, Sandoz Ltd.

Results

Prostacyclin was released from human umbilical vein endothelium. Superfusion of the endothelial cells showed that basal release of 6-oxo-PGF$_{1α}$ at the start of an experiment was 100–200 pg ml$^{-1}$ per million cells. The perfusate was changed to one containing selected concentrations of hCGRP for 4 min intervals. There was a dose-dependent increase in the release of 6-oxo-PGF$_{1α}$. Figure 1a shows this release in one of four similar experiments while Figure 1b shows the mean release of 6-oxo-PGF$_{1α}$ above basal levels in these four experiments. A positive control stimulation with bradykinin (1 μM) was included in each experiment, and previous experiments have shown that this concentration of bradykinin produces a maximum response. The response to hCGRP shows a lag of 4 min from the estimated time of arrival of peptide to the cells in the column. The release of 6-oxo-PGF$_{1α}$ mediated by 1 μM hCGRP was about 10 times less than the maximum stimulation produced by bradykinin.

In further experiments it was shown that repeated 4 min pulses of hCGRP, with 15 min of control perfusate between each, produced stimulations of 6-oxo-PGF$_{1α}$ production which were a little greater with each sequential stimulus (Figure 2).

The effect of a short 4 min stimulation was compared with that of a subsequent longer 25 min perfusion with hCGRP 10$^{-6}$ M (Figure 3a). The level of 6-
experiments different perfusion at a peak narrow varying tions of availability patterns Similar elial cells from rapid tachyphylaxis suggesting by cyclic higher a membranes prepared again pared with the contrasted the reduced and rapid 4 bradykinin of tration Eadie Hofstee linear interaction bimolecular The increase perfusate.

oxo-PGF₆₈ was greater after the long perfusion compared with the short perfusion, but the levels decreased again despite continued exposure to the hCGRP in the perfusate. The increase in 6-oxo-PGF₆₈ in the superfusate during the longer perfusion with hCGRP 10⁻⁶ M contrasted with the reduced levels of 6-oxo-PGF₆₈ seen when a long perfusion of bradykinin followed a short perfusion at the same concentration (Figure 3b). Similar patterns of 6-oxo-PGF₆₈ levels were seen in different experiments using bradykinin at concentrations varying from 10⁻⁶ to 10⁻⁸ M. Only a single narrow peak was seen with each bradykinin stimulus, suggesting rapid tachyphylaxis to its effects. Limited availability of the substrate or oxidation of the enzyme by cyclic endoperoxides were unlikely to account for the reduced peak size during the long perfusion with bradykinin 4 × 10⁻⁹ M, since a subsequent exposure to a higher concentration of Bk (10⁻⁶ M) produced a rapid and greater increase in the superfusate concentration of 6-oxo-PGF₆₈.

The activation of adenylate cyclase by hCGRP in membranes prepared from cultured human endothelial cells from 21 cords is shown in Figure 4a. The linear Eadie Hofstee plot (Figure 4b) suggests a simple bimolecular interaction between hCGRP and a single receptor population. The concentration for half max-

Figure 2 6-oxo-Prostaglandin F₆₈ (6-oxo-PGF₆₈) release from endothelial cells in consecutive volumes of perfusate following stimulation by human calcitonin gene-related peptide (hCGRP) 10⁻⁶ M (× 3) (stippled squares on horizontal axis) then bradykinin 10⁻⁷ M (solid square on horizontal axis). One of three similar experiments is illustrated.

Figure 3 (a) 6-oxo-Prostaglandin F₆₈ (6-oxo-PGF₆₈) released from endothelial cells in consecutive volumes of perfusate following replacement of the perfusion medium with one containing human calcitonin gene-related peptide (hCGRP) 10⁻⁶ M, for 4 min then for 22 min (stippled bars on horizontal axis) then for 4 min with bradykinin 10⁻⁷ M (solid square on horizontal axis). (b) 6-oxo-PGF₆₈ release from endothelial cells in consecutive volumes of perfusate following replacement of the perfusion medium with one containing bradykinin 4 × 10⁻⁹ M for 4 min then 26 min, then for 4 min with bradykinin 10⁻⁷ M (indicated on horizontal axis by solid squares and bar)
maximum enzyme activation ($K_{max}$) is given by $-1/slope$ and was 190 nM ($n = 3$). The increase in adenylate cyclase activity ($\Delta V$) with increasing concentration of hCGRP was saturatable, with a $\Delta V_{max}$ value of 9.5 pmol cyclic AMP min$^{-1}$ mg$^{-1}$ protein. In similar experiments there was no significant bradykinin-dependent enzyme activation (Figure 4a).

The rate of $[^3P]$-cyclic AMP synthesis in the absence or presence of 1 $\mu$m hCGRP is shown in Figure 5. In the presence of hCGRP the accumulation of $[^3P]$-cyclic AMP was non-linear whereas in the absence of CGRP accumulation was linear.

The relative paucity of human endothelial cells allowed only two repetitions of experiments shown in Figures 4 and 5. The results obtained were very similar.

**Discussion**

Activation of adenylate cyclase by CGRP has been demonstrated in homogenates of spleen and myocardium (Sirgrist et al., 1986). In these experiments homogenates were prepared from the heterogeneous cells of these tissues, which does not allow precise anatomical localization of the receptors. This point has been partly resolved now by the demonstration of CGRP responsiveness in cultured human endothelial cells.

The interaction of hCGRP with endothelium produces at least two distinct biological responses.
First, there is potent activation of adenylate cyclase to levels 3.5 fold above basal activity. Secondly, the vasodilator prostaglandin, prostacyclin, is released in significant amounts and in a concentration-dependent manner. The present paper confirms that bradykinin is also a potent stimulus to prostacyclin release, with levels increased by more than 20 fold. There is, however, no bradykinin-dependent activation of adenylate cyclase at concentrations up to 1 μM. This suggests that prostacyclin release and the activation of adenylate cyclase seen in response to hCGRP are not causally related.

The prostacyclin responses of endothelial cells to hCGRP are different from those to bradykinin, qualitatively as well as quantitatively. The reduction in the magnitude of the response during a prolonged infusion of bradykinin suggests that there may be development of tachyphylaxis. Although there is tachyphylaxis with prolonged perfusion of hCGRP, this is not seen with the short pulses. The hCGRP-induced peaks of 6-oxo-PGF_1α are broader than that seen with bradykinin, so a more prolonged effect of CGRP on the endothelial cells is suggested and supported by the rising baseline observed during the short pulsed stimulations with CGRP. A high affinity of binding to its receptor in conjunction with a steep dose-response curve (Figure 1b) may account for the small increases in the response to pulses of hCGRP. If some of the peptide remained bound to its receptors during the wash out, the subsequent pulses would effectively form a cumulative dose-response curve. Results of the prolonged effects of CGRP on forearm blood flow (McEwan et al., 1986) would support this interpretation. Alternatively, the initial concentration of hCGRP might be lowered by binding of the peptide to the tubing and the glass beads despite the presence of albumin in the perfusate.

The concentrations of hCGRP used in our studies were much greater than those required to produce vasodilatation in vitro or in vivo (Brain et al., 1985). This may be because the biological response of vasodilatation becomes maximal at concentrations at the lower end of the response curve for adenylate cyclase activation or release of prostacyclin. Alternatively, hCGRP-dependent vasodilatation may be unrelated to changes in cyclic AMP concentrations or prostacyclin release. However, the time course of cyclic AMP production is not linear in the presence of CGRP, and this would lead to an over-estimate of the value of $K_{av}$. The non-linearity of the time course means also that the Eadie-Hofstee plot should be interpreted with caution, since linearity is assumed in this transformation. The decrease in adenylate cyclase activity during the 30 min incubation in the presence of hCGRP, with loss of linearity, is probably due to degradation of the peptide by proteolytic enzymes despite the addition of aprotinin to the reaction mixture. The small amounts of human endothelial membranes available required that losses of the membranes were kept to a minimum during the centrifugation steps. As a result, only one washing cycle was possible in these experiments. We have shown previously that cyclic AMP generation in response to hCGRP was linear over 40 min in bovine endothelium where membranes were washed three times (unpublished observation).

Brain et al. (1985) showed that indomethacin had no effect on CGRP-induced vasodilatation in rabbit skin in vivo, and it was proposed that vasodilatation due to CGRP was independent of prostaglandin release. In contrast, the initial flare produced by intradermal injection of CGRP in man is inhibited by aspirin (Barnes et al., 1987) and it has also been shown that vasorelaxation by CGRP of rat aortic strips, preconstricted by noradrenaline, is partially inhibited by indomethacin (Brain et al., 1985). This may reflect the fact that endothelium is not uniform. Indeed, endothelial cells from microvessels have a quite different profile of prostaglandin production from those derived from large vessels (Gerritsen & Cheli, 1983; Charo et al., 1984). The studies on the vasodilatation in rabbit skin examined the effect of CGRP on arterioles while the rat aortic strip is a preparation made from a large conduit vessel and its endothelium. The present study has used endothelial cells from a large human vein. Clearly, qualitative variations in endothelium from different species and from different vessels may exist and account for conflicting results.

hCGRP is a potent vasodilator in vivo or in vitro, and the relaxation of vascular smooth muscle is dependent on the presence of an intact vascular endothelium. Endothelial cells are available for activation by hormones in the blood, and are adjacent to vascular smooth muscle cells. The vasodilator properties of hCGRP may therefore be mediated, at least in part, by vasodilator factors including prostacyclin, which are released from vascular endothelium.

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References


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