Endothelin stimulates short circuit current in a cultured epithelium

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- 1 Endothelin, a novel potent vasoconstrictor peptide produced by vascular endothelial cells stimulated anion secretion by a cultured secretory epithelium derived from the rat epididymis as measured by changes in short-circuit current (SCC).
- 2 Stimulation of the SCC was observed when endothelin was added to the basolateral or the apical side of the epithelium. The response to basolateral application was greater than that to apical application. The EC_{50} values were found to be 1.3 nM and 3.0 nM for basolateral and apical application, respectively. These values were about half an order to one order of magnitude higher than that required for its vasoconstrictor action.
- 3 The stimulation of SCC by endothelin was accompanied by a rise in the intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) content in epididymal monolayers.
- 4 The effect of endothelin on SCC was mediated through an increase in prostaglandin synthesis by the tissues and was not blocked by an antagonist of angiotensin II ($Sar^1 Ile^8$ angiotensin II) or of adrenaline (propranolol).
- 5 It is speculated that endogenous endothelin plays an important role in the control of water and electrolyte transport in the epididymis.

Introduction

Yanagisawa *et al.* (1988) have recently isolated endothelin, a potent vasoconstrictor peptide from cultured endothelial cells. These authors proposed that this peptide may mediate the endothelium-dependent vasoconstrictor action of various chemical and physical stimuli such as noradrenaline, thrombin, hypoxia, etc. (Rubanyi & Vanhoutte, 1985; Vanhoutte, 1987). The amino acid sequence has been determined and from this it appears that endothelin does not belong to any previously known peptide family. However, endothelin shows local homologies to a certain group of peptide neurotoxins that act on voltage-dependent Na channels (Yanagisawa *et al.*, 1988).

It is known that various vasoactive peptides such as vasoactive intestinal peptide (Schwartz *et al.*, 1974), angiotensin (Levens *et al.*, 1981), bradykinin (Cuthbert & Margolius, 1982) and atrial natriuretic peptide (Mohrmann *et al.*, 1987) do have effects on electrolyte and fluid transport in epithelia, and because there is evidence that endothelin acts on ion channels in cell membranes (see Yanagisawa *et al.*, 1988), we have studied the effect of endothelin on electrogenic anion secretion by a cultured epithelial monolayer derived from the rat epididymis. This epithelium has been shown to secrete anions electro-

genically when stimulated with secretory agonists that increase the level of intracellular cyclic AMP (Cuthbert & Wong, 1986; Wong, 1988a, b, c).

Methods

Measurement of short circuit current (SCC)

Primary monolayer cultures of rat cauda epididymis were grown on millipore filters by methods described previously (Cuthbert & Wong, 1986; Wong, 1988a, b, c). After 4 days of culture, the monolayer became confluent and was ready for the measurement of electrogenic anion secretion by the short circuit current technique. Monolayers (area 0.4 to 0.7 cm²) were clamped between the two halves of the Ussing chambers with a 0.7 cm² window. The tissue was short circuited (voltage clamped at zero potential) using W-P Instruments Dual Voltage Clamp Amplifier (DVC 1000) and the short circuit current displayed on a pen recorder (Kipp and Zonen). Very often, the two channels of the amplifier were used simultaneously on parallel monolayers so that studies could be made under control and experimental conditions. At the end of the experiments, the

area of the monolayers was accurately measured with an IBM computer equipped with a digitizer (Hipad™). In most experiments, monolayers were incubated in Krebs-Henseleit (K-H) solution (Wong, 1988a). In some experiments, a Cl-free solution was used in which NaCl, KCl and CaCl₂ were replaced with the corresponding gluconate salts. Endothelin was from Auspep, Australia and was reconstituted in dilute acetic acid and kept below -20°C before use. Diphenylamine-2-carboxylate (DPC) was purchased from Riedel-deHaën, W. Germany, piroxicam and prostaglandin E₂ were from Sigma, Co. (St Louis, MO, U.S.A.), lysylbradykinin from Cambridge Research Biochemicals, Cambridge, U.K., adrenaline was obtained from David Bull Laboratories, angiotensin II from Ciba Geigy, Switzerland and Sar¹, Ile⁸, angiotensin II from Beckman Instruments Inc., California, U.S.A.

Measurement of intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP)

Epididymal monolayers were grown on 24-well plates (Linbro, Flow Lab. Inc., U.S.A.). After 4 days of culture, monolayers were washed twice with Krebs Henseleit (K-H) solution and then incubated in 0.5 ml of the same buffer containing isobutylmethylxanthine (IBMX 1 mM) for 10 min at 32°C. Secretory agonists were added to the wells and incubated for a further 10 min. At the end of the incubation, 10 µl perchloric acid (60% w/v) was added to the well to terminate the reactions. The content of each well was mixed well and transferred to a 1.5 ml microfuge tube which was then centrifuged at 10,000 *g* for 5 s; 300 µl of the supernatant was transferred to a test tube and the excess acid was neutralized by 1 M KOH. Aliquots (50 µl) of the mixture were assayed for cyclic AMP with a cyclic AMP assay kit (Amersham, Buckinghamshire, U.K.). This method is based on the competition between unlabelled cyclic AMP and a fixed quantity of the tritium-labelled compound for binding to a protein which has a high specificity and affinity for cyclic AMP.

Statistical analysis

Comparisons between groups of data were made by Student's *t* test.

Results

Effect of endothelin on short circuit current

When incubated in normal Krebs-Henseleit solution, the epididymal monolayers exhibited a potential dif-

ference (PD) of 2–4 mV, a short circuit current of 1–2 µA cm⁻² and a transepithelial resistance of about 500 Ω cm² (Wong, 1988c). Addition of endothelin to the basolateral side caused a dose-dependent increase in the SCC (Figures 1 and 2). A maximal response (Δ SCC, 3.20 ± 0.12 µA cm⁻², mean $\pm$ s.e.mean, *n* = 30 cultures) was obtained with 8 nM endothelin. The EC₅₀ value (concentration producing 50% maximal response) was 1.3 nM (geometric mean, *n* = 5, 95% confidence limits = 1.19, 1.41). Addition to the apical side produced a smaller response (maximal response, 1.18 ± 0.05 µA cm⁻², *n* = 30 cultures). The EC₅₀ value was 3.0 nM (geometric mean, *n* = 5, 95% confidence limits = 2.40, 3.60).

Effects of chloride removal and diphenylamine-2-carboxylate

The short circuit current response to endothelin was studied in the absence of extracellular Cl (Cl replaced by gluconate). When monolayers were incubated in K-H solution containing Cl, the SCC response (Δ SCC) to endothelin (8 nM, basolateral application) was 3.17 ± 0.14 µA cm⁻² (*n* = 4 cultures). In monolayers incubated in Cl-free solution, Δ SCC was 0.24 ± 0.02 µA cm⁻² (*n* = 4). The difference is significant statistically (*P* < 0.001, Student's unpaired *t* test). In some experiments, monolayers were stimulated with endothelin (8 or 16 nM) added to the basolateral bathing medium. When the SCC had reached the peak level, diphenylamine-2-carboxylate (DPC, 0.1 mM) added to the apical bathing solution lowered the SCC to or below the prestimulated level (4 experiments) (Figure 1).

Effect of repeated stimulation with endothelin

The response of the tissue to apical application of endothelin can be due to the presence of receptors in the apical membrane or to the diffusion of endothelin to the basolateral side where they interact with basolateral membrane receptors. To investigate these possibilities, endothelin (1 nM) was first added to the basolateral side to produce approximately the same response as apical application of 8 nM endothelin (Figure 2). When the response subsided, endothelin (8 nM) was again added to the basolateral side to determine whether the first response had caused tachyphylaxis to the second one (Figure 3a). These results were compared with those obtained in parallel monolayers in which the tissues were first challenged with apical application (8 nM) followed by

Figure 1 Short circuit currents (SCC) of 6 separate monolayers, area 0.4–0.7 cm². Endothelin was added to the apical (○) and then the basolateral sides (●) to produce the following concentrations: (a) 0.8, (b) 1.6, (c) 4, (d) 8, and (e) 16 nM. In (f), the tissue was first challenged with endothelin (16 nM) added to the basolateral side (●) followed by apical application of diphenylamine-2-carboxylate 0.1 mM, (○). Transient current pulses were the result of intermittently clamping the potential at 0.2–0.3 mV. The horizontal lines indicate zero SCC. Each record is representative of 5 different experiments.

basolateral application (8 nM) (Figure 3b). After the tissue had first responded to basolateral application of 1 nM endothelin, the second response (Δ SCC measured at the peak of the response) to basolateral application of 8 nM endothelin was $0.90 \pm 0.05 \mu\text{A cm}^{-2}$ ($n = 4$) (Figure 3a). This

Figure 2 The effect of endothelin on the short circuit current (SCC) of rat epididymal monolayers, area 0.4–0.7 cm². Δ SCC is the increase in current measured at the peak of the response. Endothelin was added to the basolateral (●) or apical bathing medium (○). Each point shows the mean of 5 experiments; vertical lines indicate s.e.mean.

Figure 3 Short circuit currents (SCC) of 2 separate monolayers, area 0.4 cm² measured simultaneously. (a) Monolayer first stimulated with endothelin (1 nM, ●) added to the basolateral side followed by basolateral addition (8 nM, ●); (b) monolayer first stimulated with endothelin (8 nM, ○) added to the apical side followed by basolateral application (8 nM, ●). Transient current pulses were the result of intermittently clamping the potential at 0.3 mV. The horizontal lines indicate zero SCC. These records are representative of 4 different sets of experiments.

response was significantly ($P < 0.001$, Student's unpaired t test) lower than that observed in control monolayers (derived from the same batch of cells) which were treated with basolateral application of endothelin (8 nM) for the first time (Δ SCC, $3.18 \pm 0.16 \mu\text{A cm}^{-2}$, $n = 4$). This indicated that repeated basolateral application caused tachyphylaxis of the basolateral endothelin receptor. In contrast, however, in tissues that had been stimulated first with 8 nM endothelin added apically, the second response (Δ SCC) to basolateral addition of 8 nM endothelin was $3.17 \pm 0.22 \mu\text{A cm}^{-2}$ ($n = 4$). This value is not significantly different from the responses obtained from control monolayers without pretreatment with apical endothelin ($P > 0.95$, Student's unpaired t test) suggesting that apical application did not result in tachyphylaxis of the basolateral endothelin receptors.

In another group of experiments, monolayers were first treated with 8 nM endothelin to induce tachyphylaxis of the basolateral endothelin receptors, the effects of other secretory agonists on the short circuit current were then tested. Figure 4 shows that although the response to the second basolateral application of endothelin (8 nM) was prevented by tachyphylaxis of the basolateral endothelin receptors, the responses to bradykinin, prostaglandin and adrenaline were unaffected under these circumstances. This would support the contention that the endothelin receptors are distinct from those of bradykinin, prostaglandin and adrenaline (Figure 4).

Effects of prostaglandin synthesis inhibitor

The rise in SCC stimulated by endothelin was completely blocked by pretreating the tissues with an

Figure 4 Short circuit currents (SCC) of 3 separate monolayers, area 0.7 cm^2 . In each case, endothelin (8 nM) was first added to the basolateral side. When the current returned to the basal level, the tissue was challenged with endothelin for the second time followed by lysylbradykinin (Lbk $0.1 \mu\text{M}$) (a), prostaglandin E_2 (PG $0.5 \mu\text{M}$) (b), or adrenaline (Ad $0.23 \mu\text{M}$) (c) added to the basolateral side. Transient current pulses were the result of intermittently clamping the potential at 0.3 mV . The horizontal line indicates zero SCC. Each record is representative of 3 different experiments.

inhibitor of prostaglandin synthesis, piroxicam ($5 \mu\text{M}$) (Figure 5 and Table 1), indicating that the stimulation as mediated by prostaglandins synthesized locally in the tissue. In contrast, stimulation of the SCC by adrenaline was not affected by piroxicam pre-treatment (Figure 5 and Table 1).

Effects of antagonists on secretion induced by endothelin and other agonists

Previous work in our laboratory has shown that Cl secretion by epididymal epithelium is stimulated by basolateral application of angiotensin II and adrenaline (Wong, 1988a), and therefore the effects of

Figure 5 Effects of piroxicam (Piro) (prostaglandin synthesis inhibitor) on the short circuit current (SCC) response to endothelin (End) and adrenaline (Ad) in an epididymal monolayer, area 0.6 cm^2 . Monolayer first stimulated with endothelin (End) (8 nM) and then with adrenaline (Ad) ($0.23 \mu\text{M}$) both added to the basolateral side. After washing, both agents were added again in the presence of piroxicam ($5 \mu\text{M}$) added to the basolateral side. Finally, control responses were repeated after washing. Transient current pulses were the results of intermittently clamping the potential at 0.3 mV . The horizontal line indicates zero SCC. This record is representative of 3 different sets of experiments.

antagonists of angiotensin II (Sar^1 , Ile^8 Angiotensin II) and adrenaline (propranolol) were studied. Angiotensin II (500 ng ml^{-1}) added basolaterally caused an increase in the SCC that was completely abolished by Sar^1 , Ile^8 angiotensin II ($5 \mu\text{g ml}^{-1}$), an angiotensin II antagonist. However, pretreating the monolayers with the antagonist ($5 \mu\text{g ml}^{-1}$) did not affect the SCC response to endothelin (8 nM) (Table 1). Similarly, pretreating the tissues with propranolol ($2 \mu\text{M}$) did not affect the endothelin response although the same dose of propranolol almost completely blocked the SCC response to adrenaline ($0.23 \mu\text{M}$) (Table 1) (Wong & Chan, 1988).

Secretory response to endothelin mediated by cyclic AMP

Evidence has been obtained that stimulation of anion secretion in the epididymis is mediated by

Table 1 Effect of angiotensin II antagonist (Sar^1 , Ile^8 Ang II, $5 \mu\text{g ml}^{-1}$), propranolol ($2 \mu\text{M}$) and piroxicam ($5 \mu\text{M}$) on the angiotensin II-Ang II, 500 ng ml^{-1}), endothelin (8 nM) or adrenaline ($0.23 \mu\text{M}$) induced rise in the short circuit current (SCC)

Agent	ΔSCC	Number of experiments
Ang II	3.70 ± 0.15	6
Ang II + Sar^1 , Ile^8 Ang II	0^*	6
Endothelin	2.52 ± 0.27	4
Endothelin + Sar^1 , Ile^8 Ang II	2.54 ± 0.28	4
Adrenaline	10.32 ± 1.70	4
Adrenaline + propranolol	$0.12 \pm 0.02^*$	4
Endothelin	2.81 ± 0.16	4
Endothelin + propranolol	2.85 ± 0.18	4
Endothelin	3.10 ± 0.20	3
Endothelin + piroxicam	0^*	3
Adrenaline	10.83 ± 0.25	3
Adrenaline + piroxicam	10.63 ± 0.28	3

Results are expressed as the change in current, ΔSCC ($\mu\text{A cm}^{-2}$) measured at the peak of the response. Inhibitors were added to the bath 2 min (Sar^1 , Ile^8 Ang II and propranolol) or 15 min (piroxicam) before addition of the agonists. All agents were added to the basolateral bathing medium. Each value shows the mean $\pm$ s.e. mean of the number of experiments shown.

* $P < 0.001$ compared to the control of the respective group (Student's unpaired t test).

Table 2 Effect of various secretory agonists on the intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) content (pmol/10⁵ cells) of epididymal monolayers derived from the rat cauda epididymis

Agents	Cyclic AMP	Number of experiments
Control	22.6 ± 2.0	16
Adrenaline (0.23 µM)	61.5 ± 5.4*	5
PGE ₂ (0.5 µM)	40.5 ± 4.8*	6
Ang II (500 ng ml ⁻¹)	40.4 ± 5.3*	6
Endothelin (16 nM)	52.7 ± 5.8*	5

Each value is the mean ± s.e.mean of the number of experiments shown.

* $P < 0.001$ compared to control (Student's unpaired t test).

intracellular cyclic AMP as the second messenger (Cuthbert & Wong, 1986; Wong, 1988a). The cyclic AMP content in epididymal monolayers incubated in various agonists was measured. Adrenaline (0.23 µM), PGE₂ (0.5 µM), angiotensin II (500 ng ml⁻¹) or endothelin (16 nM) stimulated a rise in the concentration of intracellular cyclic AMP (Table 2).

Discussion

These results show that endothelin, a novel potent vasoconstrictor substance released from the endothelium of blood vessels, increased short circuit current in cultured rat epididymal epithelium. This increase is likely to be caused by an increase in net anion secretion since the response was greatly reduced when epididymal monolayers were incubated in a Cl-free solution. Secondly, the response was inhibited by the chloride channel blocker, DPC (0.1 mM) added to the apical solution (Figure 1). This blocker has been shown to inhibit anion secretion by cultured rat epididymal epithelium and the intact epididymis of rats *in vivo* (Wong, 1988b).

The EC₅₀ value obtained in rat epididymis is about half an order to one order of magnitude higher than that required for vascular smooth muscle contraction (Yanagisawa *et al.*, 1988) but is about one order of magnitude lower than that required for stimulation of contraction in the human isolated urinary bladder (Maggi *et al.*, 1989). The receptors mediating the effect in the epididymis are distinct from those of angiotensin, bradykinin and adrenaline (Figure 4) although the effects of endothelin, bradykinin (Cuthbert & Wong, 1986) and angiotensin II (Wong, 1988a) are mediated by an increase in prostaglandin synthesis.

The cellular mechanisms that are responsible for the stimulation of anion secretion are unknown. The intracellular messenger mediating the secretory response appears to be cyclic AMP (Table 1). In the epididymal cells, anion secretion is mediated by the active uptake of Cl and HCO₃ across the basolateral membrane and exit of the anions through the apical anion channels. The uptake of anions at the basolateral membrane is accomplished by a Na-K-2Cl symport, and a pair of antiports involving Na-H exchange and Cl-HCO₃ exchange (Wong 1988a). Stimulation of secretion can be achieved by stimulation of any of these ion carriers and/or ion channels. Recent work has shown that endothelin stimulates the Na-H exchanger in the rat mesangial cells hence causing an intracellular alkalization. This phenomenon is associated with the stimulation of mitogenesis in the cells (Simonson *et al.*, 1989).

The experiments with repeated stimulation with endothelin suggest that the responses of the tissues to apical application could not have been due to diffusion of endothelin from the apical to the basolateral side. The basolateral receptors underwent tachyphylaxis upon repeated stimulation (Figure 3a). If the responses to apical application (Figure 1) were due to diffusion of endothelin to the basolateral side, then tachyphylaxis would have reduced the responses to subsequent basolateral addition. However, this was not the case (Figure 3b) suggesting that receptors for endothelin are likely to be present in both the apical and basolateral membranes but with predominance in the latter. In this respect, the disposition of endothelin receptors is unlike that of ATP and adrenaline in that the latter two receptors display apical/basolateral 'sidedness' (Wong, 1988c).

Our present results suggest that in addition to its effects on smooth muscle (Yanagisawa *et al.*, 1988; Maggi *et al.*, 1989), endothelin may also play a role in regulating electrolyte and fluid transport in the epididymis and possibly other transporting epithelia. Koseki *et al.* (1989) studied the distribution of endothelin receptors by autoradiography and receptor binding assays and found that not only are the endothelin receptors found in the arteries and heart, they are also present in many organs such as the brain, kidney, lung, adrenal gland and intestine. These findings are consistent with the notion that endothelin may play the role of a paracrine hormone which is widely distributed and subserves diverse physiological functions.

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