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Stimulation of prostaglandin production through purinoceptors on cultured porcine endothelial cells

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ATP (approx. 1–300 μM) induces dose-dependent prostacyclin secretion from perfused columns of microcarrier beads with cultured endothelial monolayers attached. The response is transient, shows little tachyphylaxis and can reach approx. 100 times control values. 2-Methylthio-ATP is more potent, ADP slightly less potent, AMP much less potent and adenosine is ineffective. These results are consistent with the presence of a purinoceptor on endothelium linked to the prostacyclin synthetic pathway.

Extracellular ATP is a potent vasoactive agent (Green & Stoner, 1950; Burnstock, 1981). Its vasodilator responses require the presence of endothelial cells (De Mey & Vanhoutte, 1981; Gordon & Martin, 1983), suggesting that a receptor for ATP exists on vascular endothelium, but direct effects of ATP on endothelial cells have not previously been demonstrated. When vascular beds are perfused with ATP, prostaglandins are released in biologically active concentrations (Needleman *et al*., 1974; Schwartzman & Raz, 1982), but the source of these prostaglandins has not been determined.

Having established that the pig aorta exhibits endothelium-dependent relaxation to ATP (Gordon & Martin, 1983), we cultured pig aortic endothelial cells on microcarrier beads, perfused columns of these beads, and found that micromolar concentrations of ATP or ADP induce a dose-dependent release of prostacyclin. We explored the structural specificity of this response by using ATP analogues as potential agonists.

As ATP and ADP can be selectively released from stimulated platelets and vascular cells (Pearson & Gordon, 1979; Forrester, 1981), and prostacyclin has powerful effects on vascular tone and platelet function, our findings suggest that the interactions of nucleotides with the endothelial surface can contribute to vasoregulation and to haemostasis.

Abbreviations used: ATP[S], adenosine 5′-[γ-thio]-triphosphate.

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Experimental

Endothelial cells (approx. 10^6) cultured from the aortas of newborn pigs (Pearson *et al*., 1978) and used at passages 4 to 12 were added to 80 ml of Dulbecco's Modified Eagle's Medium containing 20% (v/v) foetal-calf serum and 2 ml of sterile Biosilon beads (Nunc) in the silicone-treated glass flask of an intermittent stirring apparatus (Techne, Duxford, Cambridge, U.K.). The flask was gassed with air/CO_2 (19:1) and incubated at 37°C, and the suspension was stirred for 30 s every 20 min for 3–4 days. Each bead (approx. 0.2 mm diameter) then contained 30–50 cells as estimated by microscopic examination of fixed and stained preparations; measurements of DNA and protein on the beads gave similar estimates of cell density.

Microcarrier beads with cells attached were packed in the barrel of a 1 ml disposable syringe, plugged with glass wool at the tip. Each column contained about 10^5 beads (equivalent to approx. 0.5 ml packed volume). After packing the column, the syringe plunger had a length of polyethylene tubing (0.6 mm internal diameter) inserted through it and was then reinserted in the barrel. The column was perfused upwards with serum-free Hepes [4-(2-hydroxymethyl)-1-piperazine-ethanesulphonic acid]-buffered culture medium (0.2–0.5 ml/min) and then eluate was collected every minute. Radioimmunoassay of 6-oxoprostaglandin $F_{1\alpha}$ (the stable product from prostacyclin) was performed on 0.1 ml of each sample (Ager *et al*., 1982).

Nucleotide analogues were purchased commercially [Sigma (London) Chemical Co. or Boehringer–Mannheim] apart from 2-methylthio-ATP, which was a gift from Dr. N. J. Cusack, Department of Pharmacology, King's College, London.

Results and discussion

The basal rate of prostaglandin production in perfused columns of endothelial cells was usually <20 pg/10^6 cells per min and in some experiments was barely detectable (<2 pg/10^6 cells per min). These values are similar to those for unstimulated subcultured cells grown in conventional flasks (<50 ng/10^6 cells per 24 h; see Ager *et al.*, 1982). Addition of ATP to the perfusate for 2 min stimulated prostacyclin production immediately in a dose-dependent manner (Fig. 1*a*). The minimum effective concentration was approx. 1 μM and the peak response obtained with ATP concentrations of 300–1000 μM, was >1 ng/10^6 cells per min (i.e. an increase of about 100-fold over baseline). We have consistently observed stimulation of prostacyclin release in more than 80 columns challenged with 3–300 μM-ATP or analogues thereof (see below). Porcine aortic endothelial cells in culture also secrete prostaglandin E_2 (Ager *et al.*, 1982) and this was similarly stimulated by ATP (results not shown). The stimulation of prostaglandin production was not sustained when the ATP was continuously infused; Fig. 1(*b*) shows that the response to 10 μM-ATP infused for 20 min had returned to the baseline value after 5–6 min. We have already shown that endothelial cells in perfused columns respond transiently to bradykinin or thrombin (Pearson *et al.*, 1983); the maximal response to bradykinin was 2–3-fold greater than to ATP.

Endothelial prostaglandin production was also stimulated by ADP and by AMP; ADP was almost as effective as ATP but the minimum active concentration for AMP was approx. 30 μM (see Fig. 2*a*). Adenosine had no effect even at concentrations >100 μM. Testing other nucleotides under the same conditions revealed that CTP had little effect even at concentrations of 100 μM; GTP and UTP stimulated prostaglandin production when added at 100 μM but had little effect at lower concentrations (results not shown).

Synthetic analogues of ATP were also tested as potential stimulants. Adenosine 5′-[β,γ-methylene]-triphosphate was ineffective, whereas adenosine 5′-[β,γ-imido]triphosphate was nearly as effective as ATP itself, with a threshold active concentration of <10 μM. ATP[S], which is resistant to degradation by ATPase, was slightly more effective than ATP; Fig. 2(*b*) shows that the total endothelial prostaglandin release induced by 10 μM-ATP[S] was 1.5-fold greater than the response to 10 μM-ATP, which preceded it. In eight endothelial columns challenged with 10 μM-ATP[S] under comparable conditions the peak response was 1350 ± 180 (mean ± S.E.M.) pg/10^6 cells per min. 2-Methylthio-ATP was more potent than ATP and induced prostaglandin release when perfused at 0.3 μM.

The concentrations of ATP that stimulate endothelial prostacyclin production are comparable with those previously shown to be vasoactive (Burnstock, 1981; De Mey & Vanhoutte, 1981; Gordon & Martin, 1983). It is appropriate to consider how such concentrations of extracellular ATP (or ADP) might arise. Since intracellular ATP concentrations are >1 mM, even a modest fractional release can result in biologically active concentrations extra-

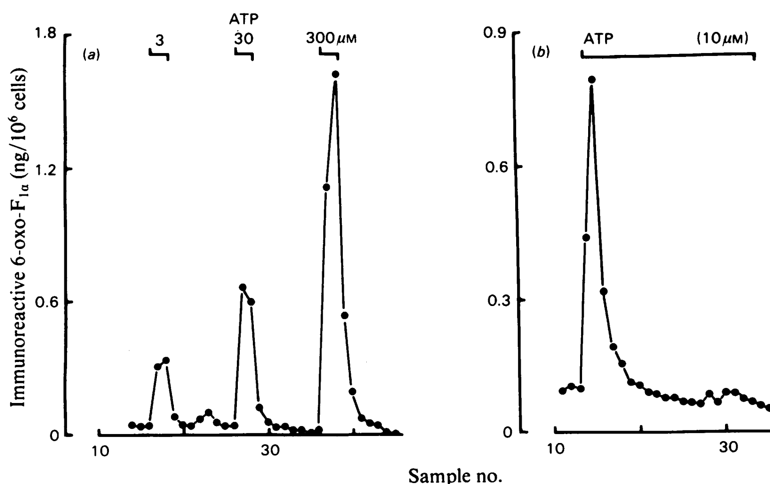

Fig. 1. *Stimulation of endothelial prostacyclin production by ATP*
(*a*) shows dose-dependent release of prostacyclin [measured as 6-oxoprostaglandin $F_{1\alpha}$ (6-oxo-$F_{1\alpha}$)] on adding ATP to the perfusate of a column containing porcine aortic endothelial cells on microcarrier beads. Samples were collected at 1 min intervals; ATP doses were added for 2 min at the points indicated. (*b*) shows that release of prostacyclin was not sustained when ATP was continuously infused for the period indicated.

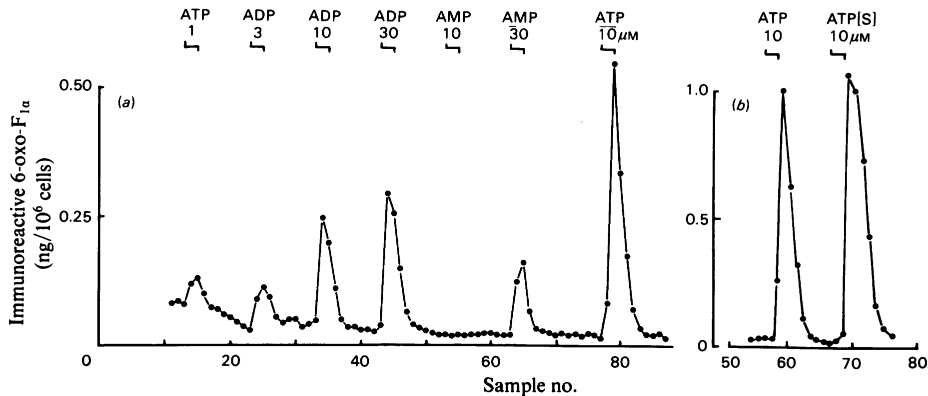

Fig. 2. *Stimulation of endothelial prostacyclin production by adenine nucleotides*
(*a*) shows a comparison of adenine nucleotides as stimulators of prostacyclin release. Experimental design was as described for Fig. 1. Abbreviation used: 6-oxo-$F_{1\alpha}$, 6-oxoprostaglandin $F_{1\alpha}$. (*b*) shows a comparison of ATP and ATP[S] as stimulators of prostacyclin release.

cellularly. There is ample evidence of ATP release into the circulation from the working heart or exercising muscle (Forrester, 1981) and vascular endothelial and smooth-muscle cells in culture can be stimulated to release ATP without affecting cell viability (Pearson & Gordon, 1979), generating extracellular concentrations high enough to exert biological effects. Furthermore, stimulated platelets can generate >10μM concentrations of ATP and ADP in plasma.

Regarding the biological significance of the prostacyclin production stimulated by ATP, the prostacyclin level in plasma is normally <3pg/ml (Blair *et al.*, 1982) and prostacyclin inhibits platelet aggregation at concentrations ⩾50pg/ml. The peak responses to 10μM-ATP we observed were about 1ng/ml, and the perfused piglet lung can release >10ng/ml in response to a bolus injection of ATP (Hellewell & Pearson, 1983).

The characterization of purinoceptors is still incomplete, but the endothelial responses are consistent with the purinoceptor type designated P_2 by Burnstock (1981): ATP is active but adenosine is not, and the 2-methylthio-analogue of ATP is more effective than ATP. ATP[S] has apparently not been tested previously as a P_2 agonist. Adenosine 5′-[β,γ-imido]triphosphate was much more active than adenosine 5′-[β,γ-methylene]triphosphate, which agrees with previous estimates of the efficacy of these two compounds as ATP analogues in other biological systems, and may be attributable to the close similarities of the P–N–P bond length and angle (but not those of the P–C–P bond) to those of the P–O–P bond (Larsen *et al.*, 1969).

The perfused column technique was used with endothelial cells by Davies (1981) to measure prostaglandin release induced by ionophore A23187, but without studying the kinetics; the sensitivity of the technique for detecting transient responses was first exploited by Busch & Owen (1982) to examine the consequence of thrombin binding to endothelium. This sensitivity stems partly from the high cell-to-medium ratio that can be achieved and partly because samples can be taken frequently without disturbing the cells. We have observed that changing the medium above endothelial cells in conventional static cultures induces more prostaglandin release than a further hour of undisturbed incubation; thus it is difficult to measure responses to stimuli unless these responses are substantial and prolonged. Weksler *et al.* (1978) failed to detect stimulation by ADP of endothelial prostaglandin production in convential cultures, and we measured only modest and inconsistent responses to ATP (even with concentrations >100μM) under these conditions. Using the perfused column technique, we have also measured responses to bradykinin and thrombin that were not detectable in cultures of the same endothelial cells studied under conventional conditions (Pearson *et al.*, 1983).

Our present results suggest that endothelium is responsible for the prostaglandins released during perfusion of vascular beds with ATP (Needleman *et al.*, 1974; Schwartzman & Raz, 1982). Extracellular ATP is catabolized by endothelial ecto-nucleotidases to adenosine, which is then transported into the cells (Pearson *et al.*, 1978, 1980). Taken together, these findings focus attention on the endothelial pericellular environment in the regulation of homeostatic processes, and on the potential role of ATP in vasoregulation.

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