Characterization of ectonucleotidases on vascular smooth-muscle cells

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1. We compared the properties of the ectonucleotidases (nucleoside triphosphatase, EC 3.6.1.15; nucleoside diphosphatase, EC 3.6.1.6; 5'-nucleotidase, EC 3.1.3.5) in intact pig aortic smooth-muscle cells in culture with the properties that we previously investigated for ectonucleotidases of aortic endothelial cells [Cusack, Pearson & Gordon (1983) *Biochem. J.* **214**, 975–981]. 2. In experiments with nucleotide phosphorothioate diastereoisomers, stereoselective catabolism of adenosine 5'-[β -thio]triphosphate, but not of adenosine 5'-[α -thio]triphosphate, by the triphosphatase and stereoselective catabolism of adenosine 5'-[α -thio]diphosphate by the diphosphatase were found, as occurs in endothelial cells. 3. In contrast with endothelial ecto-5'-nucleotidase, the smooth-muscle-cell enzyme catabolized adenosine 5'-monophosphorothioate (AMPS) to adenosine: the affinity of the enzyme for AMPS was greater than for AMP, and $V_{\max}$ for AMPS was about one-sixth that for AMP. In both cell types AMPS was an apparently competitive inhibitor of AMP catabolism by 5'-nucleotidase. 4. The relative rates of catabolism of nucleotide enantiomers in which the natural D-ribofuranosyl moiety is replaced by an L-ribofuranosyl moiety were similar to those in endothelial cells. 5. No ectopyrophosphatase activity was detected in smooth-muscle cells, in contrast with endothelial cells, where modest activity is present.

We have previously described the presence of an ectoenzyme system, consisting of nucleoside triphosphatase (EC 3.6.1.15), nucleoside diphosphatase (EC 3.6.1.6) and 5'-nucleotidase (EC 3.1.3.5), that sequentially degrades extracellular ATP to adenosine at the surface of vascular endothelial cells and vascular smooth-muscle cells (Pearson *et al.*, 1980). Extracellular adenosine derivatives powerfully affect vascular tone and blood-platelet function. We therefore proposed that the endothelial ectoenzymes are involved in the regulation of these responses (for review see Pearson & Gordon, 1985), a suggestion strengthened by the discovery that the vasodilator actions of ATP are endo-

thelium-dependent and modulated via receptors on endothelial cells rather than on smooth muscle itself (DeMey & Vanhoutte, 1981; Gordon & Martin, 1983). In addition, both ATP and adenosine have been suggested to be neurotransmitters. ATP is released from nerve terminals and has been proposed as the non-cholinergic, non-adrenergic transmitter in several (including vascular) muscle beds, and both compounds can modulate the responses to other neurotransmitters (Burnstock, 1983). Thus a regulatory role for smooth-muscle-cell ectonucleotidases in neurotransmission can be postulated; indeed, the original description of the purinergic transmitter hypothesis (Burnstock, 1972) required the presence of such enzymes, although they were not envisaged as occurring as an ectoenzyme system at the smooth-muscle-cell surface.

In the present paper we have extended our investigation of smooth-muscle-cell ectonucleotidases, in particular by using enantiomeric pairs of adenine nucleotide analogues in the manner that we have previously described for endothelial cells (Cusack *et al.*, 1983), to characterize the enzymes

Abbreviations used: AMPS, adenosine 5'-monophosphorothioate; ADP[α S], adenosine 5'-[α -thio]diphosphate; ATP[α S], ATP[β S], ATP[γ S] and ADP[β S] defined similarly; p[NH]ppA, adenosine 5'-[β , γ -imido]triphosphate; p[CH₂]ppA, adenosine 5'-[β , γ -methylene]diphosphate; pp[CH₂]pA and p[CH₂]pA defined similarly; L-AMP, AMP in which the natural D-ribofuranosyl moiety is replaced by a L-ribofuranosyl moiety; other L-nucleotides defined similarly; homo-AMP, adenosine 5'-deoxy-5'-methylenephosphonate.

and to discriminate between them and smooth-muscle-cell purinoceptors.

Materials and methods

Cell culture

Endothelial cells or smooth-muscle cells were separately isolated from the aortas of newborn pigs and cultured as previously described (Pearson *et al.*, 1978; Martin & Gordon, 1983). Cells were used for experiments after 2–17 passages.

Chemicals

[2-³H]AMP (sp. radioactivity 15–30 Ci/mmol) was purchased from Amersham International. Other chemicals were from Sigma (London) Chemical Co., except for nucleotide analogues (phosphorothioates or L-nucleotides), which were synthesized as previously described (Holý & Sörm, 1971; Eckstein, 1979; Cusack *et al.*, 1983; Cusack, 1985).

Experiments

Cells grown in 16 mm-diameter wells (approx. 10⁵ cells/well) of 24-well tissue culture trays (Nunc) were used after removal of growth medium. After rinsing the cells with Dulbecco's phosphate-buffered saline containing 1.8 mM-Ca²⁺ and 0.8 mM-Mg²⁺ (Dulbecco & Vogt, 1954), replicate incubations were carried out at 37°C in the same saline containing the appropriate nucleotides. Sub-samples of the incubation solutions were removed at timed intervals and stored at –20°C until required for analysis. In most experiments, catabolism of nucleotides or nucleotide analogues was assessed directly by h.p.l.c. separation of non-extracted sub-samples, as previously described (Cusack *et al.*, 1983). [³H]AMP catabolism was assessed by t.l.c. of sub-samples in the solvent system of Pull & McIlwain (1972), as described previously (Cusack *et al.*, 1983), except that glass-backed silica-gel layers were used. After drying the plates, radioactivity in each lane was monitored directly with a Berthold model LB2842 linear analyser.

Results

Catabolism of phosphorothioate nucleotide analogues

We investigated the stereoselectivity of the ectotriphosphatase and -diphosphatase enzymes towards the *S*_p and *R*_p diastereoisomers of ATP-[αS], ATP[βS] and ADP[αS] by incubating intact cells with 100 μM substrates, and obtained results that were essentially the same as those we found previously with endothelial cells, i.e.: (1) the catabolism of analogues in which sulphur replaces an oxygen on the penultimate phosphorus atom (ATP[βS], ADP[αS]) showed marked stereoselect-

ivity, in that detectable catabolism of only one isomer was found (ATP[βS], *R*_p; ADP[αS], *S*_p); (2) there was no stereoselectivity in the catabolism of ATP[αS] to ADP[αS]; (3) the catabolized isomers in each case were broken down at about the same rate as the corresponding natural nucleotide (results not shown).

There was no detectable catabolism of either ATP[γS] or ADP[βS], where the sulphur is bound to the terminal phosphorus atom, but, in contrast with our results with endothelial cells (Cusack *et al.*, 1983), the corresponding AMP analogue (AMPS) was catabolized. Thus 100 μM-AMPS was broken down at about one-quarter the rate of AMP (Fig. 3c), and h.p.l.c. and spectrophotometric analysis confirmed that the product was adenosine. In view of this difference we examined the catabolism of AMPS in more detail.

The concentration-dependence of AMPS catabolism is shown in Fig. 1(a). *V*_{max} (~4 nmol/min per 10⁶ cells) was about one-sixth that we previously reported for AMP catabolism in these cells (see Pearson *et al.*, 1980). The *K*_m value was about 15 μM, which is substantially lower than that for AMP catabolism (230 ± 48 μM; s.e.m. from six experiments on different cell lines). It seemed likely that AMPS was catabolized by 5'-nucleotidase, firstly because there is little or no non-specific ecto-phosphohydrolase activity in this cell type (Pearson *et al.*, 1980), and secondly because AMPS has been found not to be a substrate for such enzymes (Eckstein, 1975). To check this, we carried out two further types of experiment. In the first, we investigated the ability of p[CH₂]pA to inhibit AMPS catabolism. This analogue is well characterized as a potent and selective inhibitor of 5'-nucleotidase in several cell types, and we previously found a *K*_i value against AMP catabolism in smooth-muscle cells of about 0.1 μM (Pearson *et al.*, 1980). Fig. 1(b) shows the inhibition of AMPS catabolism by p[CH₂]pA, with a *K*_i value of 0.3 μM. Secondly, incubation of AMP (40–600 μM) with smooth-muscle cells in the presence of various concentrations of AMPS demonstrated that AMPS inhibited AMP catabolism; inhibition was detectable with 10 μM-AMPS, and the pattern of inhibition was apparently competitive (Fig. 2). In a series of three experiments of this type, we obtained a mean *K*_i value of 60 μM.

In three experiments with endothelial cells, we found that AMPS, although not catabolized itself, inhibited AMP breakdown. The pattern of inhibition was apparently competitive, with a *K*_i value for AMPS of about 50 μM (results not shown). The *K*_m for AMP catabolism in this cell type is ~24 μM (Cusack *et al.*, 1983).

Two other AMP analogues, 2-methylthio-AMPS and homo-AMP, were tested as potential

Fig. 1. Catabolism of AMPS in cultured aortic smooth-muscle cells

(a) Concentration-dependence. Points are the means of two to four observations. The line of best fit was derived from direct plot analysis (Eisenthal & Cornish-Bowden, 1974). (b) Inhibition of catabolism of 60 μM-AMPS by p[CH₂]pA. Points are the means of duplicate observations.

Fig. 2. Inhibition of catabolism of AMP by AMPS in cultured aortic smooth-muscle cells

Intact cells were incubated with various concentrations of AMP in the absence (●) or presence (○) of 100 μM-AMPS. The lines of best fit were derived from direct linear plot analysis (Eisenthal & Cornish-Bowden, 1974); *V* is in fmol/min per cell.

substrates and inhibitors of the smooth-muscle 5'-nucleotidase; 100 μM-2-methylthio-AMPS, unlike AMPS, was not detectably catabolized after up to 120 min incubations with smooth-muscle cells, and was a poor inhibitor of AMP catabolism, e.g. 1000 μM-2-methylthio-AMPS inhibited breakdown of 20–100 μM-AMP by approx. 30%. Homo-AMP was neither a substrate for nor an inhibitor of the enzyme.

Catabolism of L-nucleotides

We compared the catabolism of each L-enantiomer (in which the natural D-ribose moiety is replaced by L-ribose) with the natural isomer by incubating the intact cell layers with 100 μM substrates. The initial rates of L-ATP catabolism was approximately one-quarter the rate of ATP breakdown (Fig. 3a); the product formed was predominantly L-ADP, with little L-AMP even after 180 min. Confirming this, catabolism of exogenous L-ADP was detectable, but very slow by comparison with ADP (Fig. 3b). We could not detect any catabolism of added L-AMP (Fig. 3c).

Ectopyrophosphatase activity

We previously found modest amounts of ectopyrophosphatase activity on endothelial cells by using p[NH]ppA or p[CH₂]ppA as substrates (Cusack *et al.*, 1983). In similar experiments with smooth-muscle cells we could not find any such activity: in experiments in which 100 μM-ATP was completely catabolized in <60 min, there was no detectable catabolism of either analogue (100 μM) in 180 min. However, 100 μM-pp[CH₂]pA was catabolized slowly (<15% in 180 min) to yield p[CH₂]pA, which was stable.

Discussion

The results reported here, in conjunction with those published previously (Pearson *et al.*, 1980), demonstrate that the nucleoside tri- and diphosphatase ectoenzymes of pig aortic smooth-muscle cells are similar in many respects to their

Fig. 3. Rates of catabolism of D- and L-enantiomers of ATP, ADP and AMP, and of AMPS, by ectonucleotidases of cultured aortic smooth-muscle cells

Adenine nucleotides (100 μM) were incubated with intact cells. The ordinate axis shows the concentration of substrate remaining unchanged in the supernatant, estimated for D- and L-ATP and ADP, and for AMPS, from the peak areas obtained after h.p.l.c., and for D-AMP by spectroscopic assay of the adenosine formed.

counterparts in pig aortic endothelial cells. Thus, using L-nucleotides, we found that L-ATP is well catabolized and L-ADP less well by the smooth-muscle enzymes, exactly as is the case for the endothelial enzymes. Similarly, the pattern of stereoselectivity exhibited towards phosphorothioate analogues by the smooth-muscle ecto-triphosphatase and -diphosphatase is identical with that shown by the endothelial enzymes. The apparently contrasting stereoselectivity of the triphosphatase and the diphosphatase is due to an artefact of the *S* and *R* nomenclature: in each case the favoured isomer presents the same molecular configuration to the enzyme. The existence of stereoselectivity suggests that these ectoenzymes recognize ATP or ADP respectively complexed to Mg^{2+} in a specific manner (Eckstein, 1979). Thus the reaction mechanisms for either ectoenzyme are likely to be very similar, if not identical, in both cell types, although their active-site substrate-binding properties appear to be distinct. As noted above, in both cell types L-ATP is catabolized readily, whereas L-ADP is not. Furthermore, various ATP analogues selectively inhibit ADP catabolism, but the reverse is not true (Cusack *et al.*, 1983; Pearson, 1985).

The ecto-5'-nucleotidase of aortic smooth-muscle cells exhibited clear differences from the endothelial enzyme. Its affinity for AMP (~230 μM) is about 10-fold lower in cultured smooth-muscle cells than in cultured endothelial cells, and its V_{max} is more than 20-fold higher per cell, although we do not know whether this merely reflects a higher enzyme content. It is possible that the differences in kinetic parameters between

endothelial and smooth-muscle cell ecto-5'-nucleotidases are not so great *in vivo*, because K_m and V_{max} for the endothelial enzyme are known to decline substantially on culturing the cells (Hayes *et al.*, 1979; Chesterman *et al.*, 1983), but the fact that the disparity is maintained when the cells are cultured under similar conditions *in vitro* suggests an inherent difference in enzyme properties.

In addition we found that smooth-muscle cells, but not endothelial cells, catabolized AMPS, and we confirmed that AMPS catabolism was due to 5'-nucleotidase, by inhibiting its breakdown with $p[CH_2]pA$. Rather little work has been done on the interactions of AMPS with 5'-nucleotidase, but the purified enzyme from rat heart (where the cellular source is probably the myocyte, and the K_m for AMP is 5–20 μM) appears to behave similarly to the smooth-muscle-cell enzyme, in that it catabolizes AMPS with an affinity greater than that for AMP and a V_{max} about 6–36% that of AMP (Edwards & Maguire, 1970; Naito & Lowenstein, 1985). As noted above, in previous studies of less specific phosphatases that dephosphorylate AMP, AMPS was not a substrate, but 5'-phosphorothioate nucleotides have been reported to be competitive inhibitors (Eckstein & Sternbach, 1967; Eckstein, 1975). In our experiments AMPS inhibited 5'-nucleotidase in both vascular cell types, although it is not a substrate for the endothelial enzyme.

Because of the importance of extracellular adenosine as a local signal in the induction of reactive hyperaemia, notably in the coronary bed (Berne, 1980), there has been considerable interest in, and controversy concerning, the mechanism of adenosine production and the role of ecto-5'-

nucleotidase in this process. There may be significant cellular compartmentation of adenosine turnover in the heart, and endothelial cells could play a major part under certain conditions (reviewed in Pearson & Gordon, 1985). The finding that endothelial 5'-nucleotidase, unlike either the smooth-muscle or heart enzyme, binds but does not catabolize AMPS may be a clue indicating that the enzymes are differentially regulated *in vivo*.

Finally, there have been several suggestions that the magnitude of the pharmacological response to exogenous adenine nucleotides in various smooth-muscle beds is regulated by hydrolysis of the agonists, and hydrolysis of added ATP has been directly demonstrated in the urinary bladder (Cusack & Hourani, 1984). The present study, by characterizing the properties of vascular smooth-muscle ectonucleotidases, will be useful in defining purinoceptor agonists whose activity cannot be limited by local catabolism, and hence in discriminating between the nucleotidases and purinoceptors at the smooth-muscle-cell surface.

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