Stereoselectivity of ectonucleotidases on vascular endothelial cells

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(Received 25 April 1983/Accepted 3 June 1983)

1. We have investigated the stereoselectivity of ectonucleotidases (nucleoside triphosphatase, EC 3.6.1.15; nucleoside diphosphatase, EC 3.6.1.6; 5'-nucleotidase, EC 3.1.3.5) on pig aortic endothelial cells using two classes of nucleotide analogue. 2. In experiments with nucleotide enantiomers in which the natural D-ribofuranosyl moiety is replaced by an L-ribofuranosyl moiety, the rate of catabolism of 100 μ M-L-ATP was one-fifth that of D-ATP, the rate of catabolism of 100 μ M-L-ADP was one-fifteenth that of D-ADP and there was no detectable catabolism of 100 μ M-L-AMP. 3. Each of the L-enantiomers inhibited, apparently competitively, the catabolism of the corresponding D-enantiomer; K_i values were approx. 0.6 mM, 1.0 mM and 3.9 mM for L-ATP, L-ADP and L-AMP respectively. 4. Experiments with adenosine 5'-[β , γ -imido]triphosphate and with D- and L-enantiomers of adenosine 5'-[β , γ -methylene]triphosphate revealed modest ectopyrophosphatase activity, undetectable in experiments with natural nucleotides, which was also stereoselective. 5. Use of phosphorothioate nucleotide analogues demonstrated that ATP catabolism was virtually stereospecific with respect to the geometry of the thiol group substituted on the β -phosphate: the R_p isomer was degraded, whereas there was little or no breakdown of the S_p isomer. ADP catabolism was also stereospecific with respect to the geometry of the thiol group substituted on the α -phosphate: the S_p isomer but not the R_p isomer was degraded. The geometry of thiol-group substitution on the α -phosphate had no effect on ATP catabolism to ADP. There was no detectable catabolism of analogues with thiol-group substitution on the terminal phosphate. Each of the phosphorothioate analogues that was catabolized broke down at a rate similar to that of the natural nucleotide from which it was derived. 6. These results demonstrate that the ectonucleotidases on pig aortic endothelial cells exhibit a high degree of stereoselectivity, characteristic for each enzyme, both with respect to the ribofuranosyl moiety and to the phosphate side chain.

Extracellular adenosine and adenine nucleotides have powerful and diverse effects on several biological functions. For example, they are potent

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

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mediators of vascular tone and of blood platelet aggregation (Drury & Szent-Gyorgi, 1929; Burnstock, 1978; Haslam & Cusack, 1981). The regulation of the extracellular concentrations of these compounds is therefore important. Nucleotides can be released from nerves, working muscle and damaged or hypoxic tissue, or directly into the circulation by stimulated platelets or endothelial cells (Green & Stoner, 1950; Mills *et al.*, 1968; Burnstock, 1979; Pearson & Gordon, 1979; Forrester, 1981).

Circulating nucleotides are catabolized not predominantly by blood cells or plasma enzymes (Baer & Drummond, 1968; Brashear & Ross, 1969) but apparently at the surface of the endothelium (Ryan & Ryan, 1977; Crutchley *et al.*, 1978) to yield adenosine, which is then efficiently taken up from the circulation by the endothelial cells (Pearson *et al.*, 1978; Hellewell & Pearson, 1983). We established,

by inhibition and substrate-specificity studies, that pig aortic endothelial cells in culture possess three separate ectoenzymes, namely nucleoside triphosphatase (EC 3.6.1.15), nucleoside diphosphatase (EC 3.6.1.6) and 5'-nucleotidase (EC 3.1.3.5), that sequentially degrade extracellular ATP, and obtained approximate estimates for their kinetic parameters (Pearson *et al.*, 1980). We have subsequently examined the properties of these enzymes in more detail to compare them with other nucleotide-metabolizing enzymes. We describe here the use of two classes of nucleotide analogue for this purpose: enantiomers in which the natural D-ribofuranosyl moiety is replaced by an L-ribofuranosyl moiety (Holý & Šorm, 1971) and 5'-phosphorothioate derivatives where one of the non-bridging oxygen atoms in the phosphate side chain is replaced by a sulphur atom (Eckstein, 1979), certain of which exist as S_p and R_p diastereoisomers. The results show that the ectonucleotidases exhibit a characteristic pattern of stereoselectivity with regard to the configuration of the sugar moiety and of the phosphorothioate substituent.

Ectonucleotidases are present on several cell types (Manery & Dryden, 1979), but, with the exception of 5'-nucleotidase, which has been extensively studied, there has been little investigation of these ectoenzymes, and there is no consensus as to their functions. Since the activities of ectonucleotidases contribute to the regulation of the concentrations and pattern of extracellular nucleotides (whose actions are varied and sometimes opposing) further characterization of these enzymes is desirable. The implications of our current results are not limited solely to the control of haemostasis, or vasoactivity, and the techniques we have developed are applicable to the exploration of ectonucleotidase functions in general.

Materials and methods

Cell culture

Endothelial cells were isolated from the aorta of newborn pigs of the Babraham herd and cultured as previously described (Pearson *et al.*, 1978) in Dulbecco's modification of Eagle's medium containing 10% (v/v) foetal-bovine serum and 10% (v/v) newborn-calf serum. Cells were used for experiments after 4–20 passages.

Chemicals

[2-³H]ATP, [2-³H]ADP and [2-³H]AMP (sp. radioactivity 15–30 Ci/mmol) were purchased from Amersham International. Acetate kinase (EC 2.7.2.1), pyruvate kinase (EC 2.7.1.40), and chemicals other than nucleotide analogues were from Sigma (London) Chemical Co.

Nucleotide analogues

AMPS, ADP[β S] and ATP[γ S] were purchased from Boehringer-Mannheim. p[CH₂]pA, p[CH₂]ppA, pp[CH₂]pA and p[NH]ppA were from Sigma. Other analogues were synthesized as described below.

ADP[α S] and ATP[α S] were synthesized by phosphorylation or pyrophosphorylation respectively of AMPS, as described by Eckstein & Goody (1976). The R_p and S_p diastereoisomers of each thus obtained were separated by isocratic (0.05 M- or 0.01 M-NH₄H₂PO₄) h.p.l.c. on a reverse-phase column (μ Bondapak C18; Waters Associates) as described by Cusack & Hourani (1981, 1982). The R_p and S_p diastereoisomers of ATP[β S] were separately synthesized enzymically from ADP[β S] by phosphorylation with the combination acetyl phosphate/acetate kinase or phosphoenolpyruvate/pyruvate kinase respectively (Eckstein & Goody, 1976; Jaffe & Cohn, 1978).

9- β -L-Ribofuranosyladenosine (L-adenosine) was synthesized from L-xylose by the method of Acton *et al.* (1964). 5'-Monophosphorylation of L-adenosine, followed by phosphorylation or pyrophosphorylation, yielded L-AMP, L-ADP and L-ATP respectively (Holý & Šorm, 1971). Similarly, methylenediphosphorylation of L-AMP yielded L-p[CH₂]ppA. All nucleotides were examined by h.p.l.c. and purified if necessary by ion-exchange chromatography on Sephadex A-25 bicarbonate. Stock solutions (usually 10 mM) were assayed spectroscopically, stored at -20°C and diluted just before use.

Experiments

Confluent endothelial cell monolayers 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 (Dulbecco & Vogt, 1954) containing 1.8 mM-Ca²⁺ and 0.8 mM-Mg²⁺, replicate incubations were carried out at 37°C in the same saline containing the appropriate nucleotides. Subsamples of the incubation mixtures were removed at timed intervals and stored at -20°C until required.

Measurement of metabolites

In most experiments, catabolism of nucleotides or nucleotide analogues was assessed directly by h.p.l.c. separation of non-extracted subsamples, using ion-exchange chromatography (linear-gradient elution with 0.01–1.0 M-KH₂PO₄ on Whatman Partisil 10 SAX) or reverse-phase chromatography (0.05 M-NH₄H₂PO₄, as described above) as appropriate. Preliminary experiments established that sample extraction was unnecessary; no contaminants that interfered with h.p.l.c. separations were present in the buffered saline or extracted from the tissue-

culture plastic. Control experiments also demonstrated that neither detectable release of intracellular adenine compounds nor measurable release of enzymic activity capable of catabolizing nucleotides occurred during incubations of endothelial-cell monolayers for up to 180 min.

In some experiments, catabolism of the natural adenine nucleotides was assessed by the addition of ^3H -labelled nucleotide (100 000–500 000 d.p.m. per well) to the incubation mixture together with the unlabelled nucleotide at a known concentration. The products were then separated by t.l.c. Subsamples (10–20 μl) were chromatographed directly, with 2–5 μl of a standard marker mixture, by one of three methods. In the first, on 20 cm-long silica-gel-coated plastic sheets impregnated with fluorescent indicator (Polygram SIL G/UV254; Macherey–Nagel), the solvent system of Norman *et al.* (1974) was used at room temperature for 3–4 h or overnight. R_F values were as follows: ATP, 0.03; ADP, 0.11; AMP, 0.37; adenosine, 0.61. This system was mainly used to monitor ^3H ADP catabolism. In the second method, the same plastic sheets were used with the solvent system of Pull & McIlwain (1972) for 90–120 min at room temperature. R_F values were: ATP, ADP and AMP, 0.00; adenosine, 0.50. This system was used to study ^3H AMP catabolism. In the third method, subsamples were separated on 10 cm-long polyethyleneimine-cellulose-coated sheets (Polygram CEL 300 PEI/UV 254; Macherey–Nagel) for 45 min at room temperature in 0.5 M-LiCl containing 1.5 M-formic acid (Randerath & Randerath, 1964). R_F values were: ATP, 0.18; ADP, 0.60; AMP, 0.93; adenosine, 1.00. T.l.c. spots were located under u.v. light and outlined in pencil. Spots from silica-gel plates were cut out and placed into plastic scintillation insert vials (Mini Polyvial; Packard), 1 ml of 0.1 M-HCl was added to elute nucleotides and nucleosides and then 3 ml of scintillation fluid (Pico-Fluor 30; Packard). Spots from cellulose plates were cut out and placed into standard glass scintillation vials, 0.6 ml of 2 M-LiCl was added to elute nucleotides and nucleosides and after 5 min 3 ml of distilled water and then 15 ml of Picofluor scintillation fluid were added. Radioactivity in each vial was determined in a Beckman LS7000 scintillation counter.

Results

Kinetic analysis of catabolism of enantiomers of ATP, ADP and AMP

Estimates of the values of K_m and $V_{\max}$ for each ectonucleotidase with its natural D-enantiomer as substrate, derived from experiments on separate endothelial cell lines, are presented in Table 1. These results, which confirm our previous estimates, were obtained by incubating ^3H -labelled nucleotides

Table 1. Kinetic parameters of endothelial-cell ectonucleotidases

Results are means $\pm$ S.E.M. for the numbers of determinations shown in parentheses.

Enzyme	Substrate	K_m (μM)	$V_{\max}$ (nmol/min per 10^6 cells)
Nucleoside triphosphatase	ATP	475 ± 80 (6)	67 ± 17 (6)
Nucleoside diphosphatase	ADP	155 ± 18 (7)	9.2 ± 0.4 (7)
5'-Nucleotidase	AMP	24 ± 5 (7)	0.80 ± 0.19 (8)

with intact cell monolayers as previously described (Pearson *et al.*, 1980).

Catabolism of the L-enantiomers of ATP, ADP and AMP was investigated by incubating each nucleotide at 100 μM with intact endothelial monolayers and analysing the supernatant medium by ion-exchange h.p.l.c. L-ATP was catabolized at approximately one-fifth the rate of ATP itself, L-ADP at approximately one-fifteenth the rate of ADP and catabolism of L-AMP was undetectable (Fig. 1). We therefore investigated the interaction of the ectonucleotidases with the L-enantiomers in more detail by testing each as a potential inhibitor of the breakdown of the natural substrate. Experiments were designed with either multiple concentrations of the L-nucleotide (for Dixon analysis) or multiple substrate concentrations (for Lineweaver–Burk analysis). Each L-enantiomer inhibited the catabolism of its natural counterpart in an apparently competitive manner (see examples in Fig. 2). K_i values were 0.6 mM, 1.0 mM and 3.9 mM for L-ATP, L-ADP and L-AMP respectively.

Ectopyrophosphatase activity

Previously we failed to find any ectopyrophosphatase activity on endothelial cells; the time course of ^3H ATP catabolism indicated that virtually all the ATP was sequentially broken down to ADP and then AMP (Pearson *et al.*, 1980). We have now detected the presence of ectopyrophosphatase by incubating 100 μM -p[CH₂]ppA with cultures of endothelial cells and analysing the supernatant medium by ion-exchange h.p.l.c. Pyrophosphatase activity was modest; in experiments in which the half-life of ATP was 10 min, that of p[CH₂]ppA was 180 min. The only detectable product was adenosine, as expected, since AMP is catabolized more rapidly than the p[CH₂]p moiety is released. The ectopyrophosphatase was stereoselective; the L-enantiomer of p[CH₂]ppA (100 μM) was catabolized at about one-third of the rate of the D-enantiomer. In this case the only product was L-AMP, which, as noted above, was not further catabolized. Pyrophosphatase activity was also

Fig. 1. Rates of catabolism of D- and L-enantiomers of ATP, ADP and AMP

Confluent endothelial monolayers (approx. 10^5 cells) were incubated with $100\text{ }\mu\text{M}$ -nucleotides. The ordinate axis shows the concentration of substrate remaining unchanged in the supernatant, estimated for D- and L- ATP and ADP from the peak areas obtained after h.p.l.c., and for D-AMP by spectroscopic assay of the adenosine formed.

Fig. 2. Inhibition of catabolism of D-nucleotides by the corresponding L-enantiomer

Double-reciprocal plots are shown (●), in the absence of L-nucleotide; (○), in the presence of 1 mM -L-nucleotide. Lines of best fit are derived from the original data, using the method of Bliss & James (1966).

detected using $p[\text{NH}]ppA$ as substrate; this compound was catabolized at a similar rate to that of $p[\text{CH}_2]ppA$.

While examining the catabolism of $p[\text{CH}_2]ppA$ we also tested $pp[\text{CH}_2]pA$. This analogue was catabolized by the ectotriphosphatase to yield $p[\text{CH}_2]-pA$; the half-life of $100\text{ }\mu\text{M}$ - $pp[\text{CH}_2]pA$ was, however, more than ten times that of ATP. Metabolism of $p[\text{CH}_2]pA$ was not detected.

Catabolism of phosphorothioate analogues of ATP, ADP and AMP

Catabolism of phosphorothioate analogues was investigated by reverse-phase h.p.l.c. analysis of the supernatant medium after incubating intact endothelial monolayers with $100\text{ }\mu\text{M}$ substrates for up to 180 min.

There was no detectable breakdown of any analogue with a sulphur atom substituted for a terminal oxygen atom (i.e. AMPS, $ADP[\beta S]$, $ATP[\gamma S]$). The catabolism of analogues in which one of the free oxygen atoms on the penultimate phosphorus is replaced by sulphur ($ADP[\alpha S]$, $ATP[\beta S]$) showed marked stereoselectivity. Only the S_p isomer of $ADP[\alpha S]$ was catabolized, yielding AMPS; there was no detectable metabolism of the R_p isomer after 120 min incubation (Fig. 3a). The rate of catabolism of the S_p isomer was similar to that of ADP itself. Experiments with $ADP[\alpha S]$ were performed with unseparated mixtures of the diastereoisomers, which are readily resolved by reverse-phase h.p.l.c. {retention times for $ADP[\alpha S]$ (S_p), $ADP[\alpha S]$ (R_p) and AMPS were 6.0, 9.0 and 11.0 min respectively}. Similar experiments with

Fig. 3. Catabolism of the diastereoisomers of ADP[α S] and ATP[α S]

In (a) 100 μ M-ADP[α S] (S_p) and ADP[α S] (R_p) were incubated together above intact monolayers (approx. 10^5 cells) of endothelial cells. In (b) 100 μ M-ATP[α S] (S_p) and ATP[α S] (R_p) were incubated together above intact monolayers (approx. 10^5 cells) of endothelial cells. Catabolism was determined by reverse-phase h.p.l.c. of sub-samples of the supernatant, monitoring absorbance at 259 nm.

ATP[β S] were carried out with individual isomers; the S_p and R_p forms cannot be separated by h.p.l.c. (reverse-phase retention time 4.0 min). Marked stereoselectivity was again observed; the S_p isomer was not detectably metabolized, whereas the R_p isomer was catabolized to ADP[β S], at a rate similar to that of ATP catabolism to ADP (results not shown).

In contrast, ATP[α S] was catabolized to ADP[α S] with no evidence of stereoselectivity [Fig. 3b; reverse-phase retention times were 5.3 and 7.7 min for ATP[α S] (S_p) and ATP[α S] (R_p) respectively]. Both isomers were catabolized at approximately the same rate as ATP itself. Subsequent catabolism of the ADP[α S] diastereoisomers formed from ATP[α S] showed the expected stereoselectivity.

Discussion

The L-ribose-containing nucleotides were introduced by Holý & Šorm (1971), who synthesized oligonucleotides from them to examine their coding properties. More recently, adenosine L-ribonucleotides and purine-substituted analogues thereof have been used to test the stereoselectivity of purinoceptors on smooth muscle and blood platelets (Cusack & Planker, 1979; Cusack *et al.*, 1979; Burnstock *et al.*, 1983). Little is known of the stereoselectivity exhibited by nucleotide-metabolizing enzymes with regard to the ribose moiety, although there is a considerable literature on the mode of action of other ribose-modified adenosine analogues because of their cytotoxicity and potential use as antineoplastic agents. From such studies it appears that the enzymes responsible for phosphorylating adenosine to ATP in general tolerate a wide range of ribose modifications (Bloch, 1975). Certain enzymes will use many of the ATP analogues thus formed in reactions in which the γ -phosphate is transferred; for example, myosin accepts analogues with a diversity of ribose modifications (Tonomura *et al.*, 1967), including L-ATP (N. J. Cusack, unpublished work). In other cases, however, such as hexokinase, the enzyme tolerates sugar modifications poorly (Hohnadel & Cooper, 1972).

In our experiments, we found that L-ATP and L-ADP were acceptable substrates for the endothelial-cell ectotriphosphatase and ectodiphosphatase respectively, although significantly poorer than the natural D-nucleotides. L-ATP was bound with similar affinity to ATP itself (as indicated by its efficiency as a competitive inhibitor of ATP catabolism), whereas its rate of breakdown was about one-fifth of that of ATP, indicating only a moderate degree of stereoselectivity with regard to the ribose moiety. L-ADP was bound to the diphosphatase with an affinity approximately four times less than that of ADP, and its breakdown rate was only one-fifteenth of that of ADP when both substrates were at 100 μ M (its maximum velocity of breakdown was therefore about one-quarter of that of ADP). 5'-Nucleotidase was extremely stereoselective: L-AMP had an affinity of less than 1% of that of AMP itself, and its breakdown rate was undetectable. Holý & Šorm (1971) also reported that L-AMP was not significantly metabolized by snake venom 5'-nucleotidase. The resistance of L-AMP to catabolism by 5'-nucleotidase is of interest in that other ribose-modified AMP analogues can be acceptable substrates; e.g. each of the 2'-deoxynucleoside 5'-monophosphates is a substrate for 5'-nucleotidase (Burger & Lowenstein, 1970).

By using analogues in which the β,γ -phosphate linkage is modified to resist catabolism (p[NH]ppA and the D- and L-enantiomers of p[CH₂]ppA) we

were able to measure a low, stereoselective, ectopyrophosphatase activity in pig aortic endothelial cells that was not detectable using ATP itself. We have previously shown that there is little or no non-specific ectophosphatase activity in these cells (Pearson *et al.*, 1980), and in experiments with p[NH]ppA there was no detectable formation of [NH₂]ppA, which can be formed from p[NH]ppA by alkaline phosphatase (Yount *et al.*, 1971).

The synthesis and use of the phosphorothioate nucleotide analogues was first promoted by Eckstein (for reviews, see Eckstein, 1975, 1979), and in particular he pointed out that ATP[α S], ATP[β S] and ADP[α S] exist as S_p and R_p diastereoisomers that could be used to examine the stereoselectivity of nucleotide-metabolizing enzymes. This property has been exploited extensively by Jaffe and others (see Jaffe & Cohn, 1978, 1979; Jaffe *et al.*, 1982, and references cited therein), who have examined the relative K_m and V_{max} values of each diastereoisomer of ATP[α S] and ATP[β S] in several ATP-utilizing reactions, notably kinases. The diastereoisomers have also been used to investigate the stereoselectivity of the ADP receptor on blood platelets (Cusack & Hourani, 1981, 1982).

Our results demonstrate that none of the endothelial-cell ectonucleotidases can catabolize an adenine nucleotide when sulphur is substituted on the terminal phosphate (i.e. AMPS, ADP[β S], ATP[γ S]). This is in agreement with the view that these compounds are in general stable to degradation by phosphatases (see Eckstein, 1975); the only exception is the finding by Edwards & Maguire (1970) that 5'-nucleotidase purified from rat heart degraded AMPS almost as well as AMP itself. It should, perhaps, be noted that the terminally substituted phosphorothioates can participate in other enzymic reactions; for example, ATP[γ S] is a good substrate for myosin and for several, but not all, kinases (Eckstein, 1975; Ngoc *et al.*, 1979; Goody & Hofmann, 1980).

Experiments with phosphorothioate analogues where sulphur is substituted on the penultimate phosphate (ADP[α S], ATP[β S]) revealed that the endothelial nucleoside diphosphatase and the triphosphatase were both apparently stereospecific: in the presence of Mg²⁺ only the S_p isomer of ADP[α S] and only the R_p isomer of ATP[β S] were catabolized. It should be noted that the contrasting stereoselectivity shown by ADP[α S] and ATP[β S] is an artefact of the S and R nomenclature – the favoured isomer in each case presents the same configuration to the enzyme. Under similar conditions the ectotriphosphatase did not discriminate between the diastereoisomers of ATP[α S].

These results appear to be slightly unusual in two respects when compared with the bulk of published work on the interaction of the phosphorothioate

diastereoisomers with other enzymes. First, although all the enzymes previously studied show stereoselectivity (usually marked) for one isomer of ATP[β S] or ADP[α S], they also usually show stereoselectivity for ATP[α S] (Frey *et al.*, 1982). Of more than fifteen enzymes examined so far only phosphofructokinase (Ngoc *et al.*, 1979) and carbamate kinase (Pillai *et al.*, 1980) have shown no obvious stereoselectivity for ATP[α S]. Secondly, the favoured diastereoisomer of ATP[β S] or ATP[α S] in a range of kinase reactions often interacts less well with the enzyme concerned than does ATP itself; K_m values increase somewhat and V_{max} values decline substantially (see e.g. Jaffe & Cohn, 1979; Cohn *et al.*, 1982; Jaffe *et al.*, 1982; Frey *et al.*, 1982). We have not carried out a kinetic analysis of the interactions of the phosphorothioates with the endothelial ectoenzymes, since without purifying the enzymes from large numbers of cells this is not practicable, but the rates of breakdown of 100 μ M-ATP[β S] (R_p), -ATP[α S] and -ADP[α S] (S_p) were very similar to those of the natural substrates. In contrast, pp[CH₂]pA, an analogue in which the α,β -phosphate bond length, angle and charge distribution are modified, was catabolized very slowly by the ectotriphosphatase.

Previous studies of the stereoselectivity of nucleotide-metabolizing enzymes have used purified enzyme preparations, whereas our experiments were performed on intact cells. This approach allows the characteristics of endothelial ectonucleotidases to be compared with those of purinoceptors, because endothelial cells (like many other cell types) exhibit receptor-mediated responses to adenine nucleotides e.g. prostaglandin production, K⁺ efflux and endothelium-dependent relaxation (Gordon & Martin, 1983a,b; Pearson *et al.*, 1983). Ectoenzymes on blood platelets have been suggested as the receptor(s) for ADP that induce aggregation and inhibit adenylate cyclase (Mustard *et al.*, 1975), and some authors have identified purinoceptors on smooth muscle with ectoenzymes (Maguire & Satchell, 1981), but experimental evidence supporting these concepts is unconvincing. There have, in addition, been suggestions that the responses of smooth-muscle preparations to purinoceptor stimulation are influenced by the activity of ectonucleotidases (Burnstock *et al.*, 1983).

The results reported in the present paper will facilitate the design of agents that selectively and stereospecifically interact with ectonucleotidases, to investigate how these enzymes regulate the concentrations of extracellular adenosine derivatives and to clarify the relationship between ectonucleotidases and purinoceptors.

N. J. C. thanks the Medical Research Council (grant G8111856SA) and the British Heart Foundation (grant 81/32) for financial support.

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