The evidence for two opposite, ATP-generating and ATP-consuming, extracellular pathways on endothelial and lymphoid cells

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Extracellular purines are important signalling molecules in the vasculature that are regulated by a network of cell surface ectoenzymes. By using human endothelial cells and normal and leukaemic lymphocytes as enzyme sources, we identified the following purine-converting ectoenzymes: (1) ecto-nucleotidases, NTP diphosphohydrolase/CD39 (EC 3.6.1.5) and ecto-5′-nucleotidase/CD73 (EC 3.1.3.5); (2) ecto-nucleotide kinases, adenylate kinase (EC 2.7.4.3) and nucleoside diphosphate kinase (EC 2.7.4.6); (3) ecto-adenosine deaminase (EC 3.5.4.4). Evidence for this was obtained by using enzyme assays with ^{3}H-labelled nucleotides and adenosine as substrates, direct evaluation of γ-phosphate transfer from [γ-^{32}P]ATP to AMP/NDP, and bioluminescent measurement of extracellular ATP synthesis. In addition, incorporation of radioactivity into an approx. 20 kDa surface protein was observed following incubation of Namalwa B cells with [γ-^{32}P]ATP. Thus two opposite, ATP-generating and ATP-consuming, pathways coexist on the cell surface, where basal ATP release, re-synthesis of high-energy phosphoryls, and selective ecto-protein phosphorylation are counteracted by stepwise nucleotide breakdown with subsequent adenosine inactivation. The comparative measurements of enzymic activities indicated the predominance of the nucleotide-inactivating pathway via ecto-nucleotidase reactions on the endothelial cells. The lymphocytes are characterized by counteracting ATP-regenerating/adenosine-eliminating phenotypes, thus allowing them to avoid the lymphotoxic effects of adenosine and maintain surrounding ATP at a steady-state level. These results are in agreement with divergent effects of ATP and adenosine on endothelial function and haemostasis, and provide a novel regulatory mechanism of local agonist availability for nucleotide- or nucleoside-selective receptors within the vasculature.

Key words: ecto-adenosine deaminase, ecto-nucleotidase, ecto-nucleotide kinase, purine metabolism.

INTRODUCTION

Important roles for extracellular purines as key signalling molecules have been established in several tissues, including the cardiovascular system, where they mediate both inflammatory (ATP, ADP) and anti-inflammatory (adenosine) effects. The appearance of nucleotides in the external milieu represents a critical component of the signalling cascades, and the proposed mechanisms include ATP/ADP efflux through the opening of channel-like pathways, vesicular exocytosis and cell lysis during organ injury [1]. Once released, ATP and/or ADP regulate hemostasis and thrombosis via activation and aggregation of platelets at sites of vascular injury [2] and mediate several inflammatory responses, including the release of the cytokines, tumour necrosis factor-α and interleukin-1β from monocytes/macrophages, and facilitation of leucocyte adhesion to the endothelium [1,3–5]. Most of these effects are mediated through G-protein-coupled P2Y receptors as well as via 'cytolytic' $P2X_7$ and other P2X receptors with intrinsic pore-forming activity [4,6,7]. The duration and magnitude of purinergic signalling is governed by nucleotide-degrading ectoenzymes. Currently known nucleotidases include members of the E-type NTP diphosphohydrolase (NTPDase) family, ecto-nucleotide pyrophosphatase/phosphodiesterase family, alkaline phosphatases and ecto-5′-nucleotidase. All of them have a broad tissue distribution [8,9].

Adenosine is an important intermediate of purine nucleotide metabolism with effects that are often opposite to ATP. Particularly, adenosine has protective effects on ischaemia-induced endothelial injury, maintains endothelial barrier function, supports vasodilatation and suppresses leucocyte adhesion to the vascular endothelium [10–12]. These anti-inflammatory effects are realized through G_s-protein-coupled adenosine receptors [6] with a non-redundant role of the cAMP-elevating A2a receptor [12]. Subsequent to signal transduction, adenosine can be transported into the cell by equilibrative or Na^+-dependent nucleoside transporters to replenish the intracellular nucleotide pool [13].

Whereas most of the current models have focused on nucleotide release, signalling and degradation/inactivation aspects, the alternative pathways have received little attention. These mechanisms may include reverse nucleotide transphosphorylation by ecto-nucleotide kinases [14–16] and ATP-mediated protein phosphorylation via ecto-protein kinase reaction [17]. In the present study, we performed a comprehensive biochemical analysis of the major purine-converting activities on endothelial and lymphoid cells, including: (i) nucleotide dephosphorylation to adenosine; (ii) further adenosine deamination; (iii) reverse ATP re-synthesis; and (iv) ecto-protein phosphorylation. These findings, in conjunction with constitutive ATP release, provide a novel insight into the regulatory mechanisms of local agonist availability for nucleotide- or nucleoside-selective receptors in the bloodstream.

EXPERIMENTAL

Cell isolation and treatment

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords as described previously [15]. The B-

Abbreviations used: EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine hydrochloride; HUVEC, human umbilical vein endothelial cells; mAb, monoclonal antibody; NBTI, nitrobenzylthioinosine; NTPDase, NTP diphosphohydrolase; PBL, peripheral blood lymphocytes; PI-PLC, phosphatidylinositol-specific phospholipase C.

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cell lymphoma line Namalwa and human leukaemic T-cell lymphoblast line Jurkat were obtained from A.T.C.C. (Manassas, VA, U.S.A.) and maintained in RPMI 1640 medium supplemented with 10 % foetal calf serum, 4 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Peripheral blood lymphocytes (PBL) were isolated from freshly drawn donor blood using Ficoll centrifugations.

Prior to the experiments, HUVEC (passages 2 and 3) and lymphocytes were washed with RPMI 1640 and incubated with exogenous purines as a suspension of either intact or disrupted cells. Disruption of the cells was achieved by sonication on ice for 30 s at medium power (Braun sonicator Labsonic U). In some experiments, the cells were pre-incubated for 30 min at 37 °C with soluble apyrase from potato (grade III, 2 units/ml) or recombinant phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* (0.5 unit/ml), and then washed twice with RPMI 1640. The integrity and viability of the cells at the end of experiments were confirmed by light-microscopy observations with a Trypan Blue-exclusion test, and at least 95 % of the cells remained viable after 60–80 min incubation in RPMI 1640 medium at 37 °C. Similar results were obtained by using propidium iodine staining followed by flow-cytometric analysis.

Interconversion of ^{3}H-labelled nucleotides and adenosine

Nucleotide-converting activities were assayed by TLC with [2,8-^{3}H]ATP (specific radioactivity 19 Ci/mmol; Sigma), [2,8-^{3}H]ADP (25.3 Ci/mmol; Sigma) and [2-^{3}H]AMP (18.6 Ci/mmol; Amersham) as appropriate substrates. The standard enzyme assay contained, in a final volume of 120 μl of RPMI 1640, $(3–5) \times 10^4$ cells, 5 mmol/l β-glycerophosphate, the indicated concentrations of the substrate with tracer ^{3}H-nucleotide (approx. 4×10^5 d.p.m.) and γ-phosphate-donating ATP/NTP (in the case of nucleotide kinase studies). Incubation times were chosen to ensure the linearity of the reaction with time, so that the amount of the converted nucleotides did not exceed 7–10 % of the initially introduced substrate. Aliquots of the mixture were applied on to Alugram SIL G/UV$_{254}$ TLC sheets (Macherey-Nagel, Düren, Germany) and separated with 2-methylpropan-1-ol/3-methylbutan-1-ol/2-ethoxyethanol/ammonia/water (3:2:6:3:5, by vol.) as a solvent [18]. ^{3}H-labelled substrates and their derivatives were visualized in UV light and quantified using a Wallac-1409 β-spectrometer, as described previously [19]. A similar approach was applied for the measurement of adenosine deaminase, where cell suspensions were incubated with [2-^{3}H]adenosine (24 Ci/mmol; Amersham), followed by separation of ^{3}H-nucleosides with 2-methylpropan-1-ol/ethyl acetate/methanol/ammonia (7:4:3:4, by vol.) [20].

Flow-cytometric analysis

Endothelial and lymphoid cells were stained by immunofluorescence with anti-CD73 monoclonal antibody (mAb) 4G4 or isotype-specific mAb 3G6 as a negative control [21]. The cells were then incubated with FITC-conjugated anti-mouse Ig (Dako, Carpinteria, Glostrup, Denmark) and analysed using an FACScan with Cell Quest software (Becton Dickinson, CA, U.S.A.).

Transphosphorylation of [γ-^{32}P]ATP with AMP/NDP and protein phosphorylation

The cells were incubated for 5 min at 37 °C in a final volume of 60 μl of RPMI 1640, containing 1 μCi of [γ-^{32}P]ATP (> 5000 Ci/mmol; Amersham) and 1 mM AMP/NDP as phosphate acceptors. The samples were rapidly centrifuged. Aliquots of the supernatant were spotted on to Polygram CEL 300 PEI sheets (Macherey-Nagel) and ^{32}P-nucleotides were separated by TLC with 0.75 M KH_2PO_4 (pH 3.5). At the same time, the cell pellet was washed with RPMI 1640, boiled for 2 min with electrophoresis sample buffer [125 mmol/l Tris (pH 6.8), 4 % SDS, 10 % glycerol, 0.006 % Bromophenol Blue, 2 % 2-mercaptoethanol], and subjected to SDS/PAGE [15 % (w/v) gel].

Generation of ecto-5′-nucleotidase polyclonal antibody and immunoblotting

A peptide corresponding to residues ^{155}ETPFLSNPGTNLVFGD of the human ecto-5′-nucleotidase was synthesized, conjugated to keyhole-limpet (*Diodora aspera*) haemocyanin and used for immunizations in rabbit. The serum obtained was tested with ELISA by using purified human placental ecto-5′-nucleotidase and further purified on a BSA-conjugated peptide affinity column (SulfoLink Kit; Pierce, Rockford, IL, U.S.A.). For Western-blot analysis, the cells were incubated on ice for 10 min with lysis buffer [10 mM Tris (pH 7.4)/0.15 M NaCl/5 mM EDTA/1 % (v/v) Triton X-100/5 μM dithiothreitol/100 μM PMSF], boiled for 5 min with electrophoresis sample buffer, and subjected to SDS/PAGE (8 % gel). After transfer of separated proteins to nitrocellulose membranes and blocking with PBS containing 5 % skimmed milk and 0.2 % Tween 20, ecto-5′-nucleotidase was detected by using purified rabbit antibody (0.1 μg/ml). The bands were visualized using horseradish peroxidase-labelled anti-rabbit Ig (Dako) and an ECL® detection kit (Amersham).

Quantification of extracellular ATP level

Lymphoid cells (1×10^6/well) were incubated in 24-well plates in the starting volume of 0.5 ml of RPMI 1640 in the absence or presence of ADP/ITP. Subsamples of the medium (50 μl) were periodically collected and assayed for ATP concentration by luciferin–luciferase luminometry (Labsystems Luminoscan 1.2–0), as described previously [22].

Radioligand-binding studies

The uptake of [^{3}H]adenosine into the cells was evaluated using a high-speed filtration technique. Jurkat (1×10^5 cells) and Namalwa (5×10^5 cells) were suspended in 200 μl of RPMI 1640, containing 10 μmol/l inhibitor of adenosine deaminase *erythro*-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA; Calbiochem, La Jolla, CA, U.S.A.) and pre-incubated for 20 min at 37 °C with or without the inhibitors of nucleoside transport nitrobenzylthioinosine (NBTI) or dipyridamole (10 μmol/l; Sigma). The association reaction was started by the addition of 10 nmol/l [^{3}H]adenosine and terminated at the desired time by the addition of 3 ml of PBS followed by rapid filtration through Whatman GF/C filters. Subsequent to washing twice, radioactivity of the filters was measured in a liquid-scintillation β-spectrometer (Wallac-1409). All values were corrected for non-specific [^{3}H]adenosine binding measured in the presence of 50 μmol/l unlabelled adenosine and it was no more than 15–20 % of the total radioligand binding.

Statistics

Statistical comparisons were made using Student's t test, and $P < 0.05$ was taken as significant. Data from kinetic experiments were subjected to computer analyses using the Michaelis–Menten equation to determine the K_m and V_{max} values. All statistical analyses were performed using GraphPad Prism™ (version 3.0; San Diego, CA, U.S.A.).

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RESULTS

Extracellular hydrolysis of ^{3}H-nucleotides

Nucleotidase activities were determined by TLC as the rate of ^{3}H-nucleotides conversion into dephosphorylated ^{3}H-metabolites (e.g. pooled ADP, AMP and nucleoside fractions in the case of [^{3}H]ATP hydrolysis). Both intact cells and their sonicated lysates were used as enzyme sources, thus allowing us to evaluate the contribution of surface-associated ecto-nucleotidases to the total pool of cellular nucleotidases. Based on the preliminary kinetic studies [15], all nucleotide substrates were used at saturating concentrations that considerably exceeded the K_m(app) values for the corresponding nucleotidases.

Figure 1 shows the rates of [^{3}H]ATP (Figure 1A) and [^{3}H]ADP (Figure 1B) hydrolysis by endothelial and lymphoid cells. Among the cells used, HUVEC exhibited the highest NTPDase activities, with [^{3}H]ADP being the preferential substrate. Relatively low nucleotidase activities were observed in the experiments with Namalwa and, especially, Jurkat cells. Furthermore, ATP- and ADP-hydrolysing activities were increased by approx. 3- and 6-fold respectively after disruption of lymphoid cells, suggesting that these nucleotidases are primarily located intracellularly rather than on the lymphoid surface. The AMP that is generated in the course of NTPDase reaction can be further degraded to adenosine by another ectoenzyme 5′-nucleotidase [8]. Use of [^{3}H]AMP as initial substrate revealed that HUVEC successfully hydrolysed AMP (although not as efficiently as ATP/ADP), whereas no adenosine formation was detected in the experiments with lymphoid cells (Figure 1C). We also measured the major ecto-nucleotidase activities in freshly isolated PBL and detected relatively low rates of [^{3}H]AMP hydrolysis (8.4 ± 0.8 nmol $\cdot$ 10^6 cells^{-1} $\cdot$ h^{-1}; $n = 30$) as compared with [^{3}H]ATP (29.4 ± 3.8 nmol $\cdot$ 10^6 cells^{-1} $\cdot$ h^{-1}; $n = 14$).

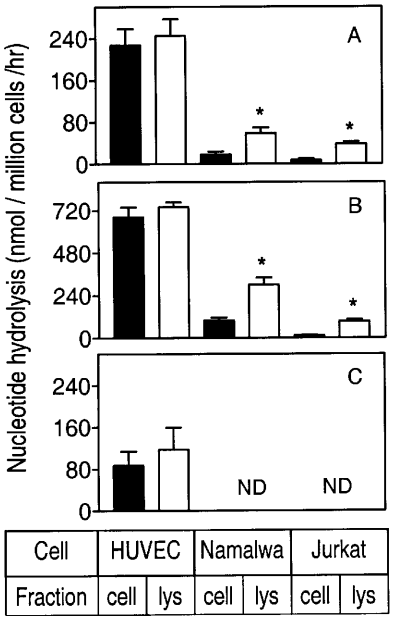

Figure 1 Nucleotide-hydrolysing activities in HUVEC and lymphoid cells

Intact cells (black bars) and their sonicated lysates (white bars) were incubated for 60 min with 800 μmol/l [^{3}H]ATP (**A**), 800 μmol/l [^{3}H]ADP (**B**) and 300 μmol/l [^{3}H]AMP (**C**). The ordinate shows enzymic activities expressed as nmol of ^{3}H-nucleotides dephosphorylated by 10^6 cells/h (means $\pm$ S.E.M.; $n = 4$–5). ND, not detectable. $^*P < 0.05$ for cell lysates as compared with the enzyme activities of intact cells.

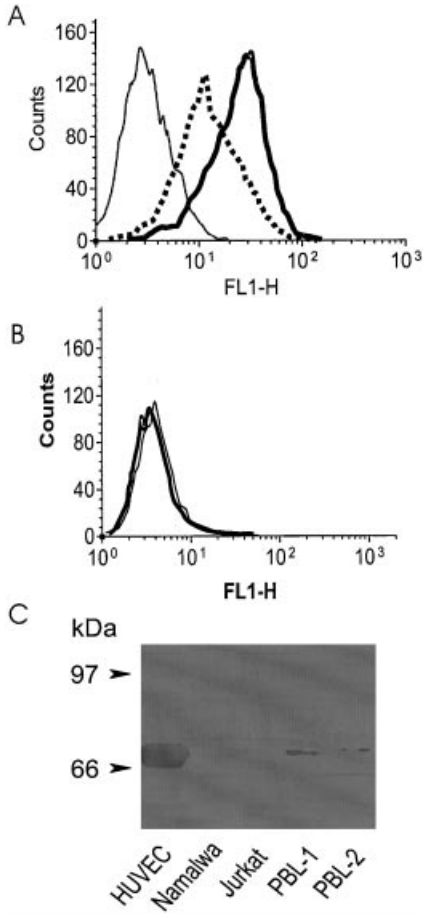

Figure 2 Ecto-5′-nucleotidase/CD73 expression on endothelial and lymphoid cells

Fluorescence histograms of HUVEC (**A**) and Jurkat cells (**B**) after incubation with an anti-CD73 mAb 4G4 (bold line) and with a negative control mAb 3G6 (thin line). CD73 expression was also detected after HUVEC treatment with 0.5 unit/ml PI-PLC (dotted line). Data are plotted as cell number versus fluorescence intensity. (**C**) Western-blot analysis of ecto-5′-nucleotidase expression in HUVEC, lymphoid cell lines Namalwa and Jurkat, as well as PBL lysates isolated from two different donors. Note that the amount of the protein loaded in the case of HUVEC and lymphocytes was equal to 15 and 50 μg respectively.

Since ecto-5′-nucleotidase activity on various lymphoid cell types has been shown to be attributed to the surface antigen CD73 [23], the relationship between ecto-5′-nucleotidase and CD73 molecules on the endothelial surface is revealed. Flow-cytometric analysis showed high expression of CD73 molecules on HUVEC surface (Figure 2A) and the complete CD73 negativity of Jurkat (Figure 2B) and Namalwa cells (results not shown). Treatment of the HUVEC with PI-PLC caused cleavage of the most glycosylphosphatidylinositol-anchored CD73 molecules and reduced the number of CD73-positive cells from $87 \pm 5\%$ ($n = 7$) to $26 \pm 3\%$ ($n = 4$) (Figure 2A). Concurrent measurement of the rate of [^{3}H]AMP hydrolysis revealed the proportional decrease of 5′-nucleotidase activity in PI-PLC-treated HUVEC by at least 75–80 % (results not shown), confirming the existence of single endothelial 5′-nucleotidase/CD73 molecule sharing both enzymic and antigenic properties.

Cell-surface expression of ecto-5′-nucleotidase/CD73 was further analysed by immunoblotting analysis with newly developed anti-ecto-5′-nucleotidase polyclonal antibody. A single

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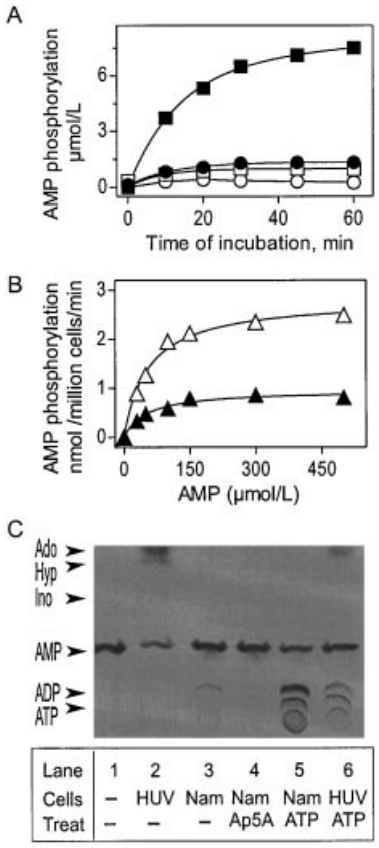

Figure 3 Pattern of AMP metabolism on the cell surface

(**A**) Namalwa cells were incubated with 10 μmol/l [^{3}H]AMP in the absence (●) and presence of 300 μmol/l ATP (■) or 50 μmol/l Ap_5A (○). The cells were also pretreated with apyrase (2 units/ml), washed and challenged with [^{3}H]AMP (□). The ordinate shows the amount of [^{3}H]AMP converted into [^{3}H]ADP/ATP (μmol/l). (**B**) Namalwa (△) and Jurkat cells (▲) were incubated with 1 mmol/l ATP in the presence of the indicated concentrations of [^{3}H]AMP. The ordinate shows adenylate kinase activity expressed as nmol of [^{3}H]ADP/ATP formed by 10^6 cells/min. (**C**) HUVEC (HUV) and Namalwa cells (Nam) were incubated for 30 min with 10 μmol/l [^{3}H]AMP in the absence and presence of 300 μmol/l ATP and 50 μmol/l Ap_5A, as indicated. [^{3}H]AMP and its ^{3}H-derivatives, ATP, ADP, adenosine (Ado), hypoxanthine (Hyp) and inosine (Ino) were separated by TLC and developed by autoradiography. Note that the amount of radioactivity in this particular illustrative experiment was 10 times higher (approx. 4×10^5 d.p.m./spot) than that used in the quantitative assays with scintillation β-counting.

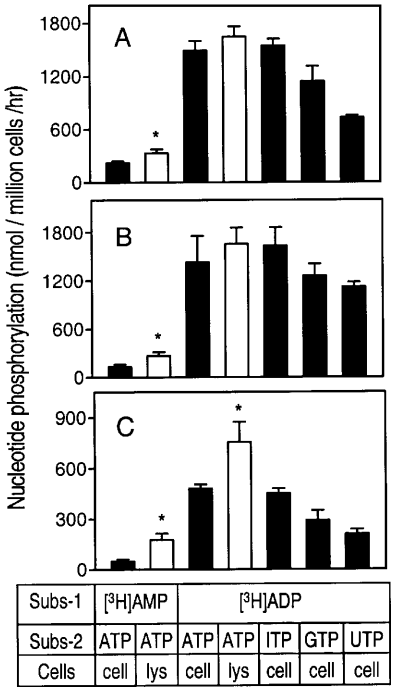

Figure 4 Nucleotide kinase activities in endothelial and lymphoid cells

HUVEC (**A**), Namalwa (**B**) and Jurkat cells (**C**) were incubated with [^{3}H]AMP (400 μmol/l; 45 min) or [^{3}H]ADP (800 μmol/l; 15 min) in the presence of 1 mmol/l ATP/NTP as phosphate donors. The ordinate shows enzymic activities expressed as nmol of ^{3}H-nucleotides phosphorylated by 10^6 cells/h (means $\pm$ S.E.M.; $n = 4$–8). Similar assays were performed with equivalent amounts of the sonicated lysates (white bars), and asterisks indicate their significant deviation from the corresponding activities of intact cells ($P < 0.05$).

protein band of approx. 70 kDa was clearly detected in HUVEC, but not in Jurkat and Namalwa cells (Figure 2C). Only a slight expression of ecto-5′-nucleotidase was detected in PBL lysates (Figure 2C), and these findings are consistent with previous results that only 11–14 % of circulating lymphocytes are CD73-positive [21,23].

Transphosphorylation of ^{3}H-labelled and unlabelled nucleotides

Namalwa cells were unable to degrade [^{3}H]AMP, but, instead, converted its minor part into ^{3}H-labelled high-energy phosphoryls (Figure 3A). Removal of extracellular ATP pool by soluble apyrase did not affect this reverse reaction, whereas specific adenylate kinase inhibitor Ap_5A prevents [^{3}H]AMP phosphorylation. Addition of exogenous ATP dramatically activated [^{3}H]AMP phosphorylation in these cells (Figure 3A), and a similar pattern of nucleotide metabolism was observed with another lymphoid cell line, Jurkat T-cells. The concentration–response curves were then generated under conditions that approximated first-order rates of the reaction, i.e. one substrate was maintained at saturating concentration (ATP; 1 mM) and the concentration of another substrate [^{3}H]AMP was varied (Figure 3B). Statistical analyses revealed that the V_{max} value for adenylate kinase reaction in Namalwa is approx. 3-fold higher than that of Jurkat cells (170 ± 6 and 58 ± 4 nmol $\cdot$ 10^6 cells^{-1} $\cdot$ h^{-1} respectively), whereas the K_m values for both cell types are comparable and vary within 50–60 μmol/l range.

While these results are principally consistent with coexistence of two opposite, ATP-consuming and ATP-generating, pathways on HUVEC surface [15], there are some clear-cut differences between endothelial and lymphoid cell types. As can be seen in Figure 3(C), addition of [^{3}H]AMP to the HUVEC caused its conversion into [^{3}H]nucleosides (lane 2), whereas Namalwa cells were unable to degrade [^{3}H]AMP (lane 3). Both lymphoid (lane 5) and endothelial (lane 6) cells caused significant ATP-mediated [^{3}H]AMP phosphorylation. However, Namalwa cells converted [^{3}H]AMP into [^{3}H]ADP even in the absence of exogenous ATP (lane 3), and this reaction was prevented by Ap_5A (lane 4).

For quantitative analysis, the cells were then incubated with [^{3}H]AMP and ATP at concentrations that significantly exceeded the K_m values for adenylate kinases in lymphoid cells (see Figure 3B) and HUVEC [15]. All cells caused specific ATP-dependent [^{3}H]AMP phosphorylation, and it was significantly increased after disruption of the cells (Figure 4). Significant adenylate kinase activity was also detected in PBL suspensions (30.6 ± 4.6 nmol $\cdot$ 10^6 cells^{-1} $\cdot$ h^{-1}; $n = 8$), which increased by 50–60 % after lymphocyte sonication. The ADP that is generated

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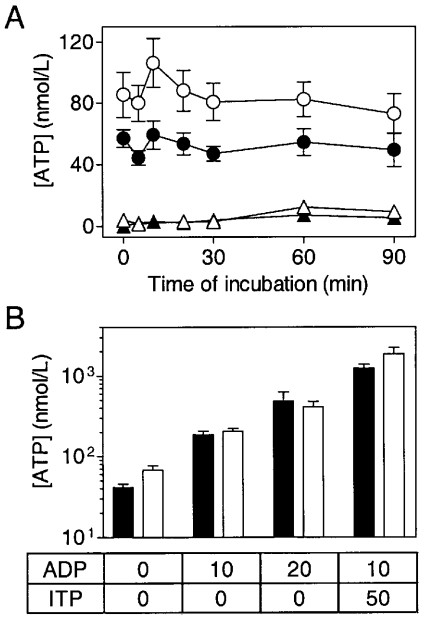

Figure 5 Bioluminescent determination of extracellular ATP

(**A**) Jurkat (○) and Namalwa cells (●) were incubated in the 24-well plates, and aliquots of the medium were assayed for ATP. Jurkat (△) and Namalwa (▲) cells were also pretreated with apyrase (2 units/ml), washed and assayed for ATP. (**B**) The ATP level was measured after 30 min incubation of Jurkat (white bars) and Namalwa (black bars) with the indicated concentrations of exogenous nucleotides (μmol/l). Note that the ordinate panel represents the logarithmic scale of ATP concentration. All values are means ± S.E.M. for at least three independent experiments.

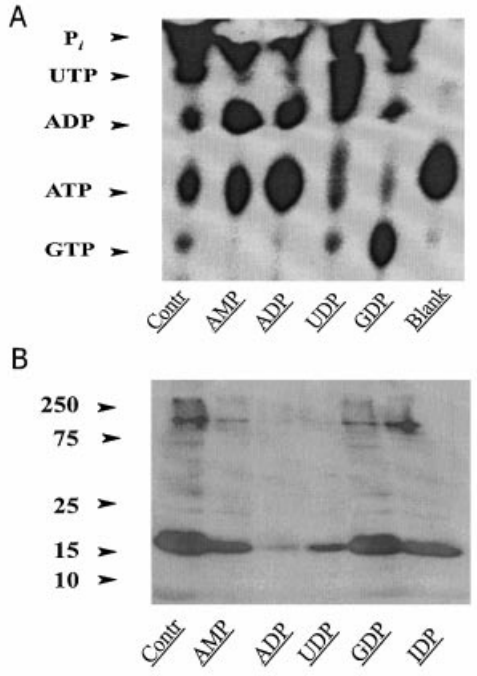

Figure 6 Transfer of γ-phosphate from [γ-^{32}P]ATP

Namalwa cells were incubated for 5 min with [γ-^{32}P]ATP in the absence (contr) and presence of 1 mmol/l AMP/NDP, as indicated. The cells were sedimented by centrifugation. Aliquots of the supernatant were loaded on to TLC plate with subsequent nucleotide separation (**A**), whereas the pellets were lysed and equal amounts of the cell protein were loaded and subjected to SDS/PAGE (15% gel) (**B**). Molecular-mass marker sizes (kDa) are indicated on the left (**B**). The last lane on autoradiography in (**A**) shows the purity of [γ-^{32}P]ATP in the absence of the cells.

in the course of adenylate kinase reaction can be further converted into ATP by ecto-NDP kinase [15], and this enzymic activity was measured with [^{3}H]ADP as an initial substrate. Unlike [^{3}H]AMP phosphorylation, subsequent [^{3}H]ADP conversion into [^{3}H]ATP is characterized by a much higher rate, is predominantly localized on the cell surface and can be activated not only by ATP but also by other γ-phosphate-donating NTPs (Figure 4).

Bioluminescent determination of extracellular ATP synthesis

To determine whether phosphoryl transfers can directly regulate the extracellular ATP level, we employed a luciferin–luciferase assay, which is highly sensitive and specific for ATP. In the absence of any treatment, the amount of ATP in the incubation medium with lymphoid cells is maintained at a certain steady-state level that can be depleted by pretreatment of the cells with apyrase (Figure 5A). Addition of 10–20 μmol/l ADP to the lymphocytes activated reverse adenylate kinase reaction with a corresponding progressive ATP formation (Figure 5B), and it can be blocked by specific adenylate kinase inhibitor Ap_5A (10 μmol/l). Addition of ADP in combination with ITP (Figure 5B) or other nucleotides, GTP and UTP (results not shown), induced even more pronounced ATP synthesis, thus confirming the necessity of a γ-phosphate donating NTP for efficient ADP conversion into ATP via ecto-NDP kinase reaction.

Metabolism of [γ-^{32}P]ATP and ecto-protein phosphorylation

Nucleotide kinase-mediated phosphotransfers were independently measured by using [γ-^{32}P]ATP. Incubation of Namalwa cells with [γ-^{32}P]ATP in the presence of 1 mmol/l AMP or NDPs caused a direct transfer of the γ-terminal [^{32}P]phosphate with the formation of the corresponding [^{32}P]ADP/NTP (Figure 6A). To test whether plasma membrane proteins can be phosphorylated under these conditions, the cell pellet was washed and subjected to SDS/PAGE. Incorporation of radioactivity into proteins was observed following incubation of [γ-^{32}P]ATP with Namalwa cells (Figure 6B), but not HUVEC or Jurkat cells (results not shown), with one approx. 20 kDa substrate being the major labelled protein. Addition of AMP and, especially, ADP/UDP together with [γ-^{32}P]ATP decreased protein phosphorylation with concurrent activation of phosphotransfers (Figure 6). Pretreatment of the cells with low concentrations of trypsin (0.5 μg or 7.2 units/sample) also markedly decreased the incorporation of radioactivity into this 20 kDa protein (results not shown), suggesting the cell-surface origin of this phosphoprotein.

Adenosine deaminase activity in endothelial and lymphoid cells

Adenosine deaminase was determined by [^{3}H]adenosine conversion into ^{3}H-labelled inosine and hypoxanthine. Substrate concentration was chosen to be 10 times that of the K_m values (approx. 70–80 μmol/l) for adenosine deaminases found in the studied lymphoid cells (results not shown) and other cell types [11]. The highest [^{3}H]adenosine deamination was observed in the experiments with Jurkat cells, which exceed the enzyme activities on HUVEC and Namalwa cells by 10- and 6-fold respectively (Figure 7). This reaction was inhibited by approx. 90% in the presence of adenosine deaminase inhibitor EHNA. Whereas disruption of the cells did not induce significant increases in the measured enzyme activities (Figure 7), such a comparative

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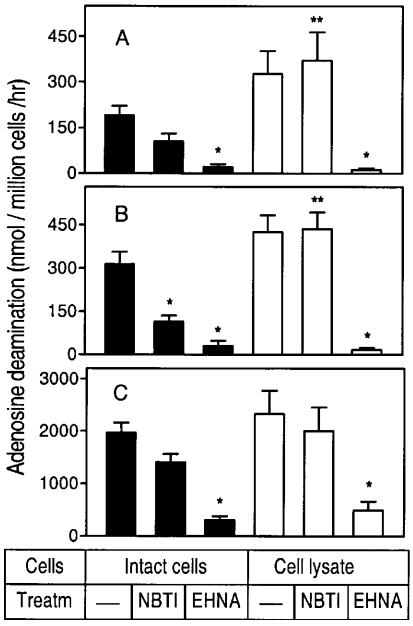


Figure 7 Adenosine deaminase activity in endothelial and lymphoid cells

HUVEC (**A**), Namalwa (**B**) and Jurkat cells (**C**) were incubated with [^{3}H]adenosine (800 μmol/l; 60 min) and the enzyme activity was determined by the formation of ^{3}H-labelled inosine/hypoxanthine. The experiments were performed with intact cells (black bars) or their sonicated lysates (white bars). The inhibitors of nucleoside transport (5 μmol/l NBTI) or adenosine deaminase (2.5 μmol/l EHNA) were added to the cells 20 min prior to the addition of [^{3}H]adenosine. $^*P < 0.05$ for NBTI- or EHNA-treated cells as compared with the corresponding activities of non-treated cells. $^{**}P < 0.05$ for cell lysates as compared with intact cells both measured under the inhibition of adenosine transport by NBTI.

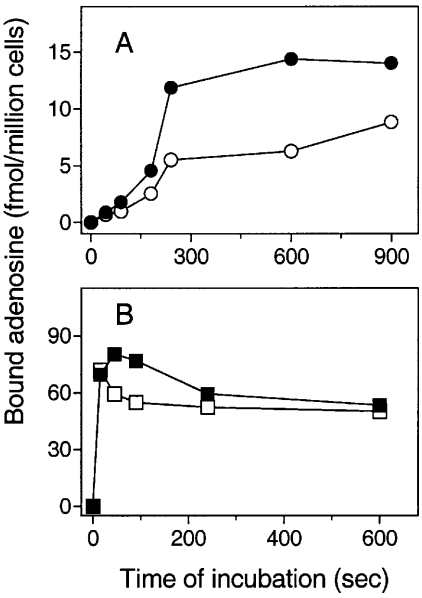


Figure 8 Time course of [^{3}H]adenosine binding to the lymphoid cells

Namalwa (**A**) and Jurkat (**B**) cells were incubated with 10 nmol/l [^{3}H]adenosine in the absence (filled symbols) and presence (open symbols) of the inhibitor of nucleoside transport NBTI (10 μmol/l). Association reaction was terminated at the indicated time points by rapid filtration of the mixture through Whatman GF/C filters. The amount of the cell-associated ligand is expressed on the ordinate as fmol of [^{3}H]adenosine per 10^6 cells. All samples contained 10 μmol/l EHNA. The graphs show mean data of two independent experiments, the results of which differed by less than 10%.

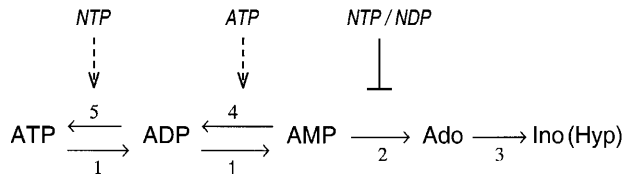
analysis is complicated by the alternative mechanism of nucleoside transport into the intact cells with subsequent intracellular deamination [13].

For direct evaluation of the extent of [^{3}H]adenosine uptake we applied the high-speed filtration technique. In order to avoid undesirable radioligand deamination, adenosine deaminase inhibitor EHNA was included in the assay buffer. [^{3}H]Adenosine binds to Namalwa cells with a pronounced lag period, and the amount of cell-associated radioligand was significantly decreased in the presence of NBTI (Figure 8A) or dipyridamole (results not shown). Contrary to Namalwa, Jurkat cells were characterized by a distinct association pattern manifested in the immediate [^{3}H]adenosine binding, about 7 times higher than the ligand-binding capacity, and insensitivity to NBTI treatment (Figure 8B). Comparison of these radioligand-binding results with enzymic results on [^{3}H]adenosine deamination by intact and disrupted cells measured in the presence of NBTI (see Figure 7) suggests that in the case of Namalwa cells (and presumably, HUVEC) most of the adenosine is transported via equilibrative nucleoside carriers with subsequent intracellular deamination, whereas an alternative inactivating mechanism via ecto-adenosine deaminase reaction prevails on the Jurkat surface.

DISCUSSION

This work was designed to evaluate the metabolism of exogenous purines by endothelial and lymphoid cells, and in this way to characterize the main ectoenzymic activities that govern the duration and magnitude of purinergic signalling in the vasculature. The following three groups of cell surface-associated enzymes were identified: nucleotide hydrolases, nucleotide kinases and adenosine deaminase. These pathways are schematically illustrated in Scheme 1.

The extracellular elements of the nucleotide-inactivating cascade that include endothelial NTPDases (otherwise known as ecto-ATPase, ATPDase, apyrase, CD39) and ecto-5′-nucleotidase/CD73 are considered to form the major effector system terminating purinergic effects of ATP/ADP in the bloodstream [24–26]. The present work, when analysed together with our previous studies [15,22], confirms the existence of high ATP/ADP-hydrolysing activities on HUVEC surface that seems to be mediated by a single ectoenzyme with hallmark characteristics of dedicated NTPDase-1 [9]. Further, AMP hydrolysis on the HUVEC surface is mediated by glycosylphosphatidylinositol-anchored ectoenzyme 5′-nucleotidase/CD73 with molecular mass of approx. 70 kDa, as verified here by its shedding from the cell surface under treatment with PI-PLC, positive staining with anti-CD73 mAbs and Western blotting with a newly developed anti-ecto-5′-nucleotidase antibody. In contrast



Scheme 1 Ectoenzymic purine turnover on the cell surface

The scheme shows the potential exchange activities of extracellular nucleotides as well as their conversion into adenosine (Ado), inosine (Ino) and hypoxanthine (Hyp). (1) NTPDase/CD39 (EC 3.6.1.5); (2) ecto-5′-nucleotidase/CD73 (EC 3.1.3.5); (3) adenosine deaminase (EC 3.5.4.4); (4) adenylate kinase (EC 2.7.4.3); (5) NDP kinase (EC 2.7.4.6). Vertical dashed arrows and blunted line depict activating and inhibiting regulatory mechanisms respectively.

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with endothelial cells with their high capacity to dispose of extracellular nucleotides, the very low (or even undetectable) activities of ecto-5′-nucleotidase/CD73 and NTPDase/CD39 have been described on various cell types of haematopoietic origin [3,24,27,28]. Our present findings with lymphoid cell lines and freshly isolated PBL are consistent with these observations.

The fate of the nucleotide-derived adenosine is defined by its uptake into the cell [13] followed by either phosphorylation to AMP via an adenosine kinase reaction or deamination to inosine by intracellular adenosine deaminase [11,29,30]. An alternative mechanism of adenosine deaminase anchorage to the plasma membrane via association with lymphocyte activation antigen CD26/dipeptidyl peptidase IV or adenosine receptors has also been demonstrated by using confocal microscopy, co-immunoprecipitation and radioligand-binding studies [31–33]. In particular, co-localization of adenosine deaminase and adenosine A_{2B} receptor has been shown recently for Jurkat T-cells [32] and our results confirmed the abundant expression of ecto-adenosine deaminase on the surface of Jurkat and, to a lesser degree, HUVEC and Namalwa cells. Furthermore, in the present study, we show that this extracellular protein is enzymically active and can be implicated, along with nucleoside transporters, in purine turnover via inactivation of external adenosine directly on the cell surface.

Along with the commonly accepted view of extracellular nucleotide degradation cascade as a purine-inactivating mechanism relevant for the function of purinergic receptors, an opposite ATP-generating pathway via phosphotransfer reactions has been only recently appreciated [14–16]. The results in the present study, together with previous competitive and substrate-specificity studies [14–16], demonstrate the expression of ecto-adenylate kinase and NDP kinase activities on the surface of endothelial and lymphoid cells. In combination, these extracellular enzymic activities would serve an anabolic purpose and maintain specific concentrations of extracellular nucleotides. Direct measurement of ATP concentration by a luciferin–luciferase assay revealed that lymphocytes maintain extracellular ATP at certain steady-state nanomolar level that represents a net balance between constitutive ATP release, and its subsequent hydrolysis and/or interconversion [34]. Furthermore, the fact that [^{3}H]AMP phosphorylation in Namalwa cells occurs even in the absence of exogenous γ-phosphate-donating ATP and was not eliminated after ATP scavenging by soluble apyrase suggests that there may be transient and local formation of relatively high (micromolar) ATP concentrations that are resistant to apyrase and different from the 'bulk' extracellular ATP pools. A similar observation has been shown previously with firefly luciferase stably adsorbed on the platelet surface [35].

We also found preferential labelling of a cell-surface-associated approx. 20 kDa substrate after the addition of [γ-^{32}P]ATP to the Namalwa cells, but not Jurkat and HUVEC. Combined exposure of the cells to ATP and other nucleotides significantly inhibited ecto-protein phosphorylation with concurrent activation of phosphotransfer reactions. These findings indicate that, in addition to serving as a substrate for ecto-nucleotide kinases, ATP can also be utilized by ecto-protein kinases and further suggest the existence of a certain competition between these ectoenzymic pathways. While the phosphorylation of cell-surface proteins and their involvement in cell–cell contacts, platelet function, cellular differentiation and proliferation are being studied vigorously [17], further studies are required to elucidate the physiological role of ecto-protein kinases and the nature of the phosphorylated proteins.

In conclusion, in the present work, we demonstrate the coexistence of two opposite ectoenzymic pathways on the cell surface, where constitutive ATP release, continuous re-synthesis of high-energy phosphoryls and selective ecto-protein phosphorylation are counteracted by stepwise nucleotide breakdown with subsequent nucleoside inactivation. Given that ecto-5′-nucleotidase catalyses the irreversible step of AMP conversion into adenosine and is strongly inhibited by precursor ADP/ATP [15,25], the specific ratios of nucleoside mono-, di-, and triphosphates determine the shift towards nucleotide synthesis or degradation. Either direction may be locally influenced by a specific enzymic make-up or by physiological circumstances. Moreover, taking into account the ability of NDP kinase to use indifferently all naturally occurring ribonucleotides, one might suggest that the crucial role of this ectoenzyme is the directional switch between various purine and pyrimidine receptors via rapid interconversion of the circulating NTP/NDP.

The relative activities of purine-converting ectoenzymes vary dramatically among endothelial and lymphoid cells, and these findings are particularly pertinent in this context, with divergent effects of ATP and adenosine in the vasculature [4,6]. In this scenario, the cells with high NTPDase and ecto-5′-nucleotidase activities, such as vascular endothelium, tend to compensate pro-inflammatory and pro-thrombotic effects of circulating ATP/ADP with respective generation of anti-inflammatory agent adenosine. In contrast, the lymphoid cells with low or no ecto-nucleotidase activities and a relatively high expression of ecto-adenosine deaminase are characterized by 'ATP-generating/adenosine-eliminating' phenotype, thus allowing them to avoid lymphotoxic effects of adenosine, impair endothelial barrier function and propagate ATP-mediated signalling effects to the endothelial cells.

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REFERENCES

1 Kunapuli, S. P. and Daniel, J. L. (1998) P2 receptor subtypes in the cardiovascular system. Biochem. J. **336**, 513–523
2 Robson, S., Sevigny, J., Imai, M., Guckelberger, O. and K, E. (2000) Thromboregulatory potential of endothelial CD39/nucleoside triphosphate diphosphohydrolase: modulation of purinergic signalling in platelets. Emerging Ther. Targets **4**, 155–171
3 Clifford, E. E., Martin, K. A., Dalal, P., Thomas, R. and Dubyak, G. R. (1997) Stage-specific expression of P2Y receptors, ecto-apyrase, and ecto-5′-nucleotidase in myeloid leukocytes. Am. J. Physiol. **273**, C973–C987
4 Di Virgilio, F., Chiozzi, P., Ferrari, D., Falzoni, S., Sanz, J. M., Morelli, A., Torboli, M., Bolognesi, G. and Baricordi, O. R. (2001) Nucleotide receptors: an emerging family of regulatory molecules in blood cells. Blood **97**, 587–600
5 MacKenzie, A., Wilson, H. L., Kiss-Toth, E., Dower, S. K., North, R. A. and Surprenant, A. (2001) Rapid secretion of interleukin-1β by microvesicle shedding. Immunity **15**, 825–835
6 Ralevic, V. and Burnstock, G. (1998) Receptors for purines and pyrimidines. Pharmacol. Rev. **50**, 413–492
7 North, R. A. and Surprenant, A. (2000) Pharmacology of cloned P2X receptors. Annu. Rev. Pharmacol. Toxicol. **40**, 563–580
8 Zimmermann, H. (1992) 5′-Nucleotidase: molecular structure and functional aspects. Biochem. J. **285**, 345–365
9 Zimmermann, H. (2000) Extracellular metabolism of ATP and other nucleotides. Naunyn Schmiedebergs Arch. Pharmacol. **362**, 299–309
10 Cronstein, B. N. (1994) Adenosine, an endogenous anti-inflammatory agent. J. Appl. Physiol. **76**, 5–13
11 Spychala, J. (2000) Tumor-promoting functions of adenosine. Pharmacol. Ther. **87**, 161–173
12 Ohta, A. and Sitkovsky, M. (2001) Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. Nature (London) **414**, 916–920
13 Griffith, D. A. and Jarvis, S. M. (1996) Nucleoside and nucleobase transport systems of mammalian cells. Biochim. Biophys. Acta **1286**, 153–181

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14 Lazarowski, E. R., Homolya, L., Boucher, R. C. and Harden, T. K. (1997) Identification of an ecto-nucleoside diphosphokinase and its contribution to interconversion of P2 receptor agonists. J. Biol. Chem. **272**, 20402–20407
15 Yegutkin, G. G., Henttinen, T. and Jalkanen, S. (2001) Extracellular ATP formation on vascular endothelial cells is mediated by ecto-nucleotide kinase activities via phosphotransfer reactions. FASEB J. **15**, 251–260
16 Donaldson, S. H., Picher, M. and Boucher, R. C. (2002) Secreted and cell-associated adenylate kinase and nucleoside diphosphokinase contribute to extracellular nucleotide metabolism on human airway surfaces. Am. J. Respir. Cell. Mol. Biol. **26**, 209–215
17 Redegeld, F. A., Caldwell, C. C. and Sitkovsky, M. V. (1999) Ecto-protein kinases: ecto-domain phosphorylation as a novel target for pharmacological manipulation? Trends Pharmacol. Sci. **20**, 453–459
18 Norman, G. A., Follett, M. J. and Hector, D. A. (1974) Quantitative thin-layer chromatography of ATP and the products of its degradation in meat tissue. J. Chromatogr. **90**, 105–111
19 Yegutkin, G. G. and Burnstock, G. (1998) Steady-state binding of [^{3}H]ATP to rat liver plasma membranes and competition by various purinergic agonists and antagonists. Biochim. Biophys. Acta **1373**, 227–236
20 Pull, I. and McIlwain, H. (1972) Metabolism of (14C)adenine and derivatives by cerebral tissues, superfused and electrically stimulated. Biochem. J. **126**, 965–973
21 Airas, L., Niemela, J., Salmi, M., Puurunen, T., Smith, D. J. and Jalkanen, S. (1997) Differential regulation and function of CD73, a glycosyl-phosphatidylinositol-linked 70-kDa adhesion molecule, on lymphocytes and endothelial cells. J. Cell Biol. **136**, 421–431
22 Yegutkin, G., Bodin, P. and Burnstock, G. (2000) Effect of shear stress on the release of soluble ecto-enzymes ATPase and 5′-nucleotidase along with endogenous ATP from vascular endothelial cells. Br. J. Pharmacol. **129**, 921–926
23 Resta, R., Yamashita, Y. and Thompson, L. F. (1998) Ecto-enzyme and signaling functions of lymphocyte CD73. Immunol. Rev. **161**, 95–109
24 Coade, S. B. and Pearson, J. D. (1989) Metabolism of adenine nucleotides in human blood. Circ. Res. **65**, 531–537
25 Meghji, P., Pearson, J. D. and Slakey, L. L. (1995) Kinetics of extracellular ATP hydrolysis by microvascular endothelial cells from rat heart. Biochem. J. **308**, 725–731
26 Enjyoji, K., Sévigny, J., Lin, Y., Frenette, P. S., Christie, P. D., Esch, II, J. S., Imai, M., Edelberg, J. M., Rayburn, H., Lech, M. et al. (1999) Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. Nat. Med. (Tokyo) **5**, 1010–1017
27 Dombrowski, K. E., Ke, Y., Brewer, K. A. and Kapp, J. A. (1998) Ecto-ATPase: an activation marker necessary for effector cell function. Immunol. Rev. **161**, 111–118
28 Koziak, K., Sevigny, J., Robson, S. C., Siegel, J. B. and Kaczmarek, E. (1999) Analysis of CD39/ATP diphosphohydrolase (ATPDase) expression in endothelial cells, platelets and leukocytes. Thromb. Haemost. **82**, 1538–1544
29 Spychala, J., Mitchell, B. S. and Barankiewicz, J. (1997) Adenosine metabolism during phorbol myristate acetate-mediated induction of HL-60 cell differentiation: changes in expression pattern of adenosine kinase, adenosine deaminase, and 5′-nucleotidase. J. Immunol. **158**, 4947–4952
30 Aldrich, M. B., Blackburn, M. R. and Kellems, R. E. (2000) The importance of adenosine deaminase for lymphocyte development and function. Biochem. Biophys. Res. Commun. **272**, 311–315
31 Franco, R., Valenzuela, A., Lluis, C. and Blanco, J. (1998) Enzymatic and extraenzymatic role of ecto-adenosine deaminase in lymphocytes. Immunol. Rev. **161**, 27–42
32 Herrera, C., Casado, V., Ciruela, F., Schofield, P., Mallol, J., Lluis, C. and Franco, R. (2001) Adenosine A2B receptors behave as an alternative anchoring protein for cell surface adenosine deaminase in lymphocytes and cultured cells. Mol. Pharmacol. **59**, 127–134
33 Gines, S., Marino, M., Mallol, J., Canela, E. I., Morimoto, C., Callebaut, C., Hovanessian, A., Casado, V., Lluis, C. and Franco, R. (2002) Regulation of epithelial and lymphocyte cell adhesion by adenosine deaminase-CD26 interaction. Biochem. J. **361**, 203–209
34 Lazarowski, E. R., Boucher, R. C. and Harden, T. K. (2000) Constitutive release of ATP and evidence for major contribution of ecto-nucleotide pyrophosphatase and nucleoside diphosphokinase to extracellular nucleotide concentrations. J. Biol. Chem. **275**, 31061–31068
35 Beigi, R., Kobatake, E., Aizawa, M. and Dubyak, G. R. (1999) Detection of local ATP release from activated platelets using cell surface-attached firefly luciferase. Am. J. Physiol. **276**, C267–C278

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