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Inhibitory effects of some purinergic agents on ecto-ATPase activity and pattern of stepwise ATP hydrolysis in rat liver plasma membranes

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Abstract

Inhibitory effects of various purinergic compounds on the Mg^{2+}-dependent enzymatic hydrolysis of [^{3}H]ATP in rat liver plasma membranes were evaluated. Rat liver enzyme ecto-ATPase has a broad nucleotide-hydrolyzing activity, displays Michaelis–Menten kinetics with K_m for ATP of 368 ± 56 μM and is not sensitive to classical inhibitors of the ion-exchange and intracellular ATPases. P2-antagonists and diadenosine tetraphosphate (Ap_4A) progressively and non-competitively inhibited ecto-ATPase activity with the following rank order of inhibitory potency: suramin (pIC_{50}, 4.570) > Reactive blue 2 (4.297) ≫ Ap_4A (3.268) > pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) (2.930). Slowly hydrolyzable P2 agonists ATPγS, ADPβS, α,β-methylene ATP and β,γ-methylene ATP as well as the diadenosine polyphosphates Ap_3A and Ap_5A did not exert any inhibitory effects on the enzyme activity at concentration ranges of 10^{-4}–10^{-3} M. Thin-layer chromatography analysis of the formation of [^{3}H]ATP metabolites indicated the presence of other enzyme activities on liver surface (ecto-ADPase and 5′-nucleotidase), participating in concert with ecto-ATPase in the nucleotide hydrolysis through the stepwise reactions ATP → ADP → AMP → adenosine. A similar pattern of sequential [^{3}H]ATP dephosphorylation still occurs in the presence of ecto-ATPase inhibitors suramin, Ap_4A and PPADS, but the appearance of the ultimate reaction product, adenosine, was significantly delayed. In contrast, hydrolysis of [^{3}H]ATP in the presence of Reactive blue 2 only followed the pattern ATP → ADP, with formation of the subsequent metabolites AMP and adenosine being virtually eliminated. These data suggest that although nucleotide-binding sites of ecto-ATPase are distinct from those of P2 receptors, some purinergic agonists and antagonists can potentiate cellular responses to extracellular ATP through non-specific inhibition of the ensuing pathways of purine catabolism.

Keywords: Liver plasma membrane; Rat; Ecto-ATPase; P2 receptor; Inhibition

Abbreviations: ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); Ap_3A, diadenosine triphosphate; Ap_4A, diadenosine tetraphosphate; Ap_5A, diadenosine pentaphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); α,β-MeATP, α,β-methylene ATP; β,γ-MeATP, β,γ-methylene ATP; *p*-CMBS, *p*-chloromercuriphenyl sulfonate; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; RB2, Reactive blue 2 (Cibacron blue 3GA); TLC, thin-layer chromatography

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1. Introduction

The great physiological importance of extracellular ATP in the organism is characterised on the one hand by its direct involvement in the control of diverse cellular functions, and on the other by regulatory role of biologically active compounds derived from subsequent nucleotide breakdown. ATP can be released into the extracellular space through the opening of channel-like pathways or via vesicular exocytosis [1–3] and can then initiate its purinergic effects in an autocrine or paracrine fashion through activation of G protein-coupled P2Y receptors or ligand-gated P2X receptors [1,3–6]. Following the initiation of biological effects, ATP is sequentially degraded to adenosine by plasma membrane nucleotidases ecto-ATPase (EC 3.6.1.15), ATP diphosphohydrolase (ecto-apyrase; EC 3.6.1.5), ecto-ADPase (EC 3.6.1.6) and ecto-5′-nucleotidase (EC 3.1.3.5) [2,7–9]. The involvement of other ecto-enzymes in the metabolism of extracellular ATP has also been described, i.e. nucleoside diphosphate kinase (EC 2.7.4.6) [7,10] and ecto-nucleotide pyrophosphatase (EC 3.6.1.9) [11]. The adenosine formed either interacts with its own P1 receptors to trigger cellular responses [5] or may be imported into the cell by specific Na^+-dependent nucleoside transporters for the recapture of nucleosides [12,13].

This model of extracellular ATP turnover can apply well to the liver. Endogenous ATP, released into the canalicular lumen from the hepatocytes and bile duct cells [14,15], interacts with nucleotide-binding sites of P2Y receptors on liver plasma membranes [16,17] to trigger various signalling events including mobilisation of extracellular Ca^{2+}, glycogen breakdown, K^+ uptake and opening of Cl^- channels [1,14–16], and is finally degraded to adenosine by canalicular ecto-ATPase and 5′-nucleotidase [13].

Although the role of ATP as a potent effector of signal transduction processes has been intensively studied, the involvement of ecto-nucleotidases in these purinergic processes remains to be clarified. Inhibition of a subsequent step of ATP hydrolysis would provide a powerful research tool to estimate the 'true' biological potency of ATP and its analogues in the tissue. However, none of the currently available purinergic agents has been found to be selective ecto-ATPase inhibitors [18–24]. Moreover, some of these P2 agonists and antagonists are capable of potentiating responses to extracellular ATP and concurrently inhibit ecto-ATPase activity [18–21]. When an additional property is inhibition of an enzyme which acts to degrade the experimental agonist, potentiation of agonist concentration effect (E/[A]) curves can occur. Combination of this property with receptor antagonism can result in 'self-cancellation' of E/[A] curve displacement in which neither the enzyme inhibitory nor receptor antagonist components are fully expressed [19,25].

To obtain deeper insight into these complex regulatory/inhibitory mechanisms, it is necessary to perform kinetic and competitive analysis of the nucleotide–receptor interaction on cell surface together with the ensuing pathways of purine catabolism. We have reported recently a steady-state analysis of native [^{3}H]ATP binding to rat liver plasma membranes and its displacement by various P2 agonists and antagonists [17]. In this present study, we have investigated some of these purinergic agents as inhibitors of liver ecto-ATPase, and discuss the impact of ectoenzymatic breakdown of ATP on the pharmacological analysis of P2 receptors from the viewpoint of agonist and antagonist action.

2. Materials and methods

2.1. Materials

[2,8-^{3}H]ATP (specific activity 40 Ci/mmol) was purchased from ICN Biomedicals (Belgium). Organic solvents were from Analar. Liquid scintillation cocktail Wallac OptiPhase 'HiSafe'-3 was from Fisher. Pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) was from Tocris Cookson. Thin-layer chromatography (TLC) plates were 20×10 cm silica gel 60 F_{254} type supplied by Sigma-Aldrich Chemicals. All other chemicals were purchased from Sigma (Poole, UK).

2.2. Liver plasma membrane isolation

Male Sprague–Dawley rats weighing 200–220 g were killed by asphyxiation with CO_2 and plasma membranes were isolated from the liver by method

of Dorling and Le Page [26] with some modifications [17]. The resulting membrane pellet was resuspended in 7 mM Tris-HCl (pH 7.4) at a concentration 2–2.5 mg protein/ml and stored in aliquots in liquid nitrogen. Plasma membrane preparations were enriched by approximately 14-fold with 5′-nucleotidase activity compared with the initial homogenate. Protein was determined by a modified Lowry method [27] using bovine serum albumin as standard.

2.3. Ecto-nucleotidase assay

Rat liver ATPase activity and the pattern of ATP hydrolysis was measured using TLC assay as the most sensitive and versatile approach for screening the catabolism of adenine nucleotides [7]. The standard assay contained in a final volume of 100 μl, 5–7 μg of membrane protein, 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 50–1000 μM ATP with tracer [^{3}H]ATP (5×10^5 dpm) and 200 μM ouabain, unless otherwise indicated. Incubation times were chosen to ensure the linearity of the reaction with time and concentration of the membrane protein. In the case of inhibitory studies the membranes were preincubated with various concentrations of inhibitors at 37°C for 10 min prior to the addition of [^{3}H]ATP. Catalytic reactions were terminated by applying aliquots of the mixture on TLC plates, and nucleotides were separated using an appropriate solvent system [28] as described previously [17]. Radioactive areas that co-migrated with respective nucleotide/nucleoside standards were scraped into scintillation vials, and radioactivity was determined using scintillation counting on a β-spectrometer (Packard, Minaxi Tricarb 4000 series).

2.4. Statistics and data analysis

Data from competitive experiments were subjected to computer analysis by non-linear least-squares curve fitting to determine IC_{50} values (GraphPad Prism software). In the case of kinetic studies, the K_m and V_{max} values were calculated using a non-linear curve fitting program based on the Michaelis-Menten equation (Enzkin, GraphPad Prism). Statistical comparisons were made using Student's *t*-test, and *P*-values <0.05 were taken as significant.

3. Results

3.1. Identification, catalytic activity and substrate specificity of rat liver ecto-ATPase

Using rat liver plasma membranes as a source of ecto-nucleotidases, we measured ATPase activity by TLC as the rate of exogenous [^{3}H]ATP conversion into [^{3}H]ADP. Liver ATPase exhibited strong dependence on the presence of Mg^{2+} or Ca^{2+} in the assay buffer, since in the presence of the chelating agent EDTA there was no measurable degradation of [^{3}H]ATP (Table 1). No significant additive effect was observed when the enzyme activity was measured in the presence of both $CaCl_2$ and $MgCl_2$ together with ATP, suggesting that a single enzyme may be activated by either Ca^{2+} or Mg^{2+}.

Table 2 illustrates that ATPase on liver membranes successfully hydrolysed all naturally occurring nucleoside triphosphates, as well as ADP, whereas the ability to hydrolyse AMP and adenosine was

Table 1
Effect of various compounds on Mg^{2+}-dependent hydrolysis of [^{3}H]ATP in rat liver plasma membranes

Compound	Concentration (mM)	% ATPase activity
Control	–	100
Ouabain	0.2	88.1 ± 2.5
$CaCl_2$	5.0	112.0 ± 7.0
EDTA	20.0	2.0 ± 1.0
DTT	1.0	96.0 ± 2.0
N-Ethylmaleimide	1.0	94.8 ± 4.4
p-CMBS	1.0	98.8 ± 1.1
NaN_3	5.0	68.2 ± 3.5
Ortho-vanadate	0.15	83.9 ± 4.6
α,β-MeATP	1.6	80.5 ± 4.5
β,γ-MeATP	1.6	85.2 ± 2.3
ATPγS	0.8	96.0 ± 3.1
ADPβS	0.8	93.0 ± 4.8
Ap_3A	2.0	86.6 ± 6.0
Ap_5A	2.0	92.0 ± 3.1

Liver plasma membranes were incubated without (control) or with the compounds indicated for 10 min at 37°C prior to the addition of 500 μM [^{3}H]ATP. Standard incubation medium is described in Section 2 except that ouabain was not included in the reaction mixture unless its effects were being studied. The results are expressed as a percent of [^{3}H]ATP hydrolysis in the treated membranes, compared with the controls. Values for controls were 16.5 ± 2.1 μmol/mg/h (mean ± S.E.M.; $n=3$).

Table 2
Substrate specificity of Mg^{2+}-dependent hydrolysis of [^{3}H]ATP in rat liver plasma membranes

Compound	% of control
[^{3}H]ATP (control)	100
ATP	59.7
GTP	64.1
ITP	72.1
UTP	65.0
CTP	82.2
ADP	61.8
AMP	98.5
Adenosine	100
β-Glycerophosphate	100

In the control situation, 500 μM of ATP with [^{3}H]ATP tracer was added to rat liver plasma membranes of which 120 ± 10 μM was hydrolysed. This 24% breakdown was defined as 100% activity. In all other cases, in addition to 500 μM [^{3}H]ATP, 1 mM of nucleotide analogue was also present. The data are the mean from the representative experiment which was repeated twice.

virtually absent. No changes of ATP hydrolysis were detected in the presence of β-glycerophosphate, indicating that non-specific phosphatases are poorly represented in the plasma membranes studied. Thus, liver ATPase recognises a subset of nucleotides only if they contain tri- or diphosphates, although amongst all the nucleotides used, ATP and ADP appeared to be preferential substrates for the enzyme activity.

Several known inhibitors of ion-exchanging and intracellular ATPases [8] were examined for their ability to inhibit Mg^{2+}-dependent [^{3}H]ATP hydrolysis in liver plasma membranes (Table 1). About 88% of the ATPase activity was ouabain-insensitive and this inhibitor of Na^+/K^+-ATPase was included in the assay buffer in further experiments. ATPase activity was weakly inhibited by sodium azide and ortho-vanadate and remained insensitive to *N*-ethylmaleimide, *p*-chloromercuriphenyl sulfonate (*p*-CMBS) and dithiothreitol.

Taken together, these data show that rat liver plasma membranes possess an ATP-metabolising enzyme with characteristics of an E-type ATPase [8,29], such as Ca^{2+} and Mg^{2+} dependence, broad nucleotide-hydrolysing activity and insensitivity to the classic inhibitors of the V-, P- and F-type ATPases. As for the partial reduction in ATPase activity by azide and vanadate, it may reflect the ability of these compounds to weakly inhibit some, but not all, ecto-apyrases and ecto-ATPases [2,8].

3.2. Competitive analysis of ATP hydrolysis by rat liver ecto-ATPase

Various non-hydrolysable nucleotide analogues were examined for their ability to inhibit ecto-ATPase activity in rat liver, including P2Y agonists adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) and adenosine 5′-*O*-(2-thiodiphosphate) (ADPβS), P2X agonists α,β-methylene ATP (α,β-MeATP) and β,γ-methylene ATP (β,γ-MeATP), and diadenosine polyphosphates Ap_3A, Ap_4A and Ap_5A which act as physiologically relevant P2 agonists. Several commonly used P2 receptor antagonists were also examined for their inhibitory effects on liver ecto-ATPase, including the trypanocidal agent suramin, the dye Reactive blue 2 (RB2) and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS). Only Ap_4A and the P2 antagonists suramin, RB2 and PPADS were able to inhibit [^{3}H]ATP hydrolysis in a concentration-dependent manner (Fig. 1), whereas other purinergic compounds studied were shown to be weak ecto-ATPase inhibitors (Table 1). The pIC_{50} values were calculated from the inhibitory curves and

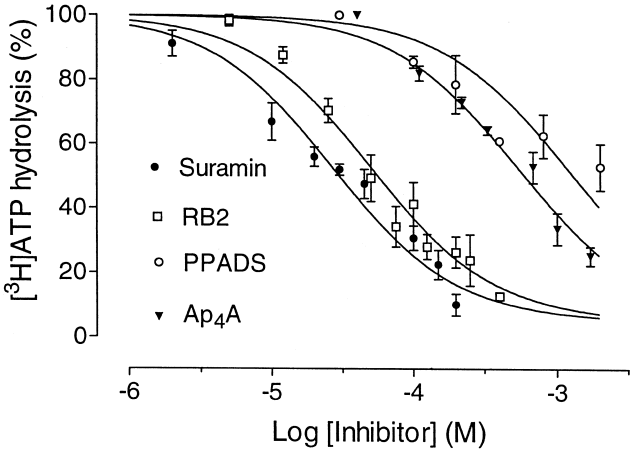

Fig. 1. Inhibition of rat liver ecto-ATPase by purinergic agents. Rat liver plasma membranes were preincubated for 10 min at 37°C with increasing concentrations of suramin, RB2, PPADS or Ap_4A prior to the addition of 500 μM [^{3}H]ATP. The results are plotted as the percentage of maximal ATP hydrolysis measured in the absence of competitors. The values for control ecto-ATPase activity were 15.0 ± 1.7 μmol/mg/h. Each point is the mean ± S.E.M. from three experiments performed in duplicate.

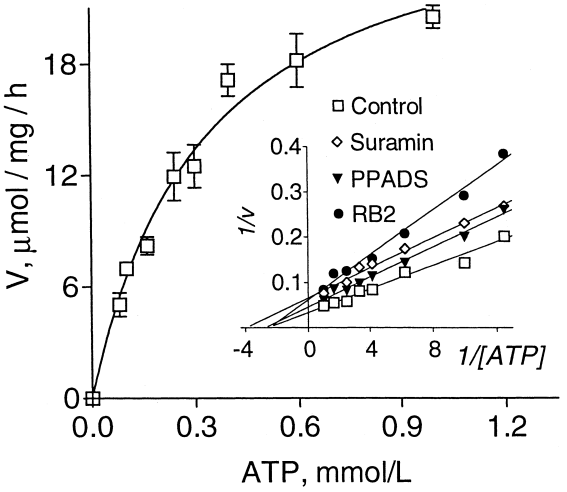

Fig. 2. Rate of [^{3}H]ATP hydrolysis by rat liver plasma membranes versus substrate concentration plot. Ordinate, liver ecto-ATPase activity expressed as μmol ATP hydrolysed by 1 mg membrane protein in 1 h. Abscissa: concentration of unlabelled ATP, mM. Inset: Lineweaver–Burk plot showing inhibition of [^{3}H]ATP hydrolysis by P2-antagonists suramin (40 μM), RB2 (50 μM) and PPADS (400 μM).

the following rank order of inhibitory potency was established: suramin > RB2 > Ap_4A > PPADS (Table 3).

To further elucidate the mechanism of ecto-ATPase inhibition by P2 antagonists, kinetic analysis of [^{3}H]ATP hydrolysis was carried out in the presence of fixed amounts of certain inhibitors. The concentrations of inhibitors were chosen to approximate to their respective IC_{50} values found in the preliminary competitive experiments (see Fig. 1). Fig. 2 shows a curvi-linear Michaelis–Menten plot describing the rate of [^{3}H]ATP hydrolysis by control plasma membranes versus unlabelled substrate concentration and a linear representation of these data according to Lineweaver–Burk. The enzyme kinetics were significantly modified in the presence of suramin, RB2 and PPADS and the double reciprocal plots of these

Table 3
Competitive inhibition of [^{3}H]ATP hydrolysis by rat liver ecto-ATPase

Inhibitor	pIC_{50}
Suramin	4.570 ± 0.037
RB2	4.297 ± 0.040
Ap_4A	3.268 ± 0.030
PPADS	2.930 ± 0.081

Hydrolysis of [^{3}H]ATP in rat liver plasma membranes was inhibited by increasing concentrations of the indicated compound. Competition curves were then constructed (see Fig. 1) and fitted to a one-site model using non-linear least-squares curve fitting. The constants are expressed in terms of pIC_{50} ($-\log IC_{50}$) ± S.E.M. ($n = 3$).

Table 4
Effect of P2 antagonists on kinetic parameters of rat liver ecto-ATPase

Inhibitor	Concentration of inhibitor (μM)	Kinetic parameters of ATPase	
		V_{max} (μmol/mg/h)	K_m (μM)
None (Control)	–	28.9 ± 2.0	368 ± 56
Suramin	40	17.6 ± 1.8[a]	454 ± 48
RB2	50	19.0 ± 2.0[a]	461 ± 97
PPADS	400	19.2 ± 1.5[a]	312 ± 50

Liver plasma membranes were incubated with [^{3}H]ATP and increasing concentrations of unlabelled ATP in the absence (control) or presence of the fixed concentration of certain inhibitors (see also Fig. 2). V_{max} and K_m values were calculated using a non-linear curve fitting program based on the Michaelis–Menten equation. Values are the mean ± S.E.M. ($n = 4$).
[a] $P < 0.05$ compared with control.

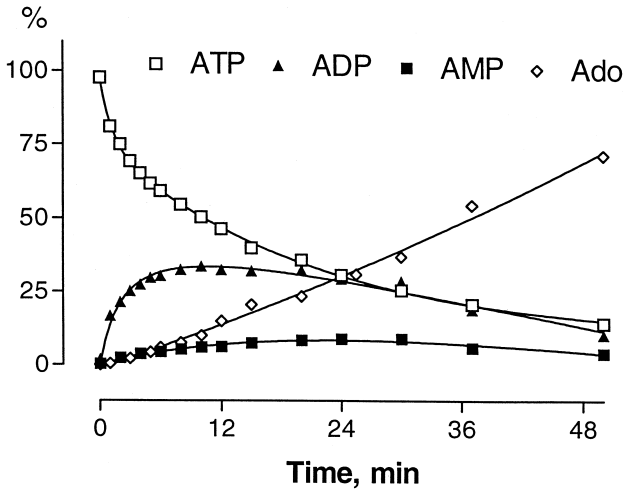

Fig. 3. Time course of the sequential ATP hydrolysis by rat liver plasma membranes. The membranes were incubated with [^{3}H]ATP and aliquots of the mixture were periodically applied on TLC sheets for separation of ATP and its metabolites (Section 2.3). Initial ATP concentration is 360 μM. Ordinate: relative content of [^{3}H]ATP and ^{3}H-labelled products of its hydrolysis, ADP, AMP and adenosine (Ado) expressed as percentage of initial concentrations. The graphs show mean data ($n = 3$); the standard error of the mean did not exceed the size of symbols.

competitive data can be seen in the inset to Fig. 2. Each of the purinergic antagonists studied significantly decreased the maximum hydrolysis rate of ecto-ATPase (V_{max}) without any modification of the enzyme affinity (K_m) (Table 4), indicating that inhibitory effects of these compounds are non-competitive in nature.

3.3. Effects of purinergic compounds on the pattern of ATP hydrolysis by liver membranes

A representative time course of [^{3}H]ATP hydrolysis by rat liver membranes is illustrated in Fig. 3. The decrease in ATP concentration with time was accompanied by an immediate rise in ADP concentration which reached maximum value after 8–12 min and then gradually decayed. Little AMP was formed, but after a time lag there was rapid production of adenosine. The levels of metabolites showed an apparent precursor–product reaction suggesting stepwise ATP dephosphorylation via ADP to AMP and adenosine.

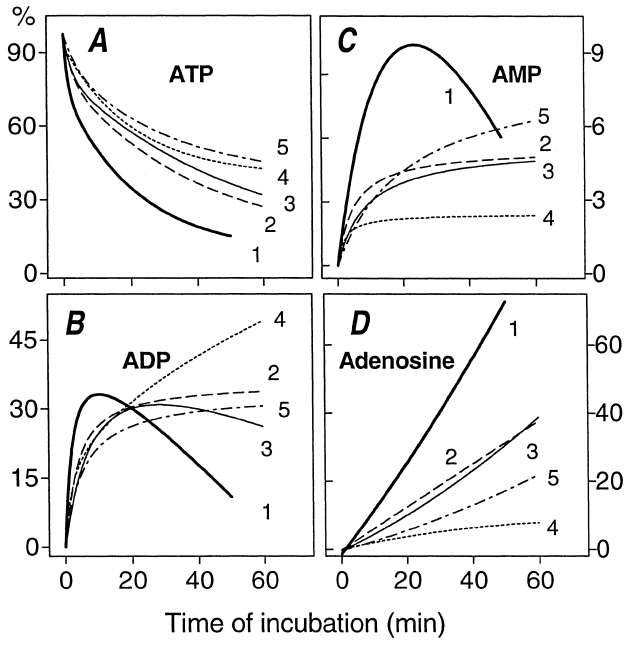

Fig. 4. Effect of purinergic agents on the pattern of ATP hydrolysis and product formation in rat liver plasma membranes. The membranes were incubated with 360 μM [^{3}H]ATP in the absence (1) or presence of 40 μM suramin (2), 1000 μM PPADS (3), 50 μM RB2 (4), 500 μM Ap_4A (5). Subsamples of the medium were collected at timed intervals (see Fig. 3) for subsequent analysis of decrease in [^{3}H]ATP (A) and formation of the dephosphorylated [^{3}H]-derivatives ADP (B), AMP (C) and adenosine (D). For clarity the symbols were omitted from the graphs. The graphs show mean data of two independent experiments.

In order to investigate whether any of the subsequent reaction rates can also be affected by ecto-ATPase inhibitors, we examined the effects of purinergic compounds on the whole kinetics of ATP hydrolysis by liver membranes. A similar pattern of stepwise [^{3}H]ATP dephosphorylation to adenosine still occurs in the presence of suramin, Ap_4A and PPADS, but the rates of appearance of ADP and AMP and their conversion into adenosine were significantly delayed with time (Fig. 4). Another compound studied, RB2, also inhibited ATP conversion into ADP (Fig. 4A). The latter nucleotide accumulated progressively in the analysed mixture, accounting for nearly 85% of the products formed 60 min after 360 μM ATP was added (Fig. 4B). The rates of appearance of the subsequent metabolites AMP (Fig. 4C) and adenosine (Fig. 4D) were practically prevented under the action of RB2.

4. Discussion

4.1. Characterisation of ecto-ATPase and other nucleotidase activities in rat liver plasma membranes

The pattern of [^{3}H]ATP dephosphorylation by rat liver plasma membranes indicates the presence of a cascade of surface-bound enzymes hydrolyzing adenine nucleotides through the stepwise reactions ATP → ADP → AMP → adenosine. Conversion of exogenous ATP to ADP appears to be mediated by ecto-ATPase, which displays the general characteristics of a dedicated E-type ATPases [8,29]. The ADP produced can be further hydrolyzed to AMP by another member of E-type ATPase family ecto-apyrase and/or ecto-ADPase. It should be noted that nucleotide pyrophosphatases/phosphodiesterases are also expressed on the liver membranes [30,31] and can be involved in the degradation of extracellular ATP. However, direct conversion of ATP into AMP did not occur to any significant extent in our experiments. Another enzyme participating in the observed process of nucleotide degradation is ecto-5′-nucleotidase, hydrolyzing AMP to adenosine in the

studied membrane preparations with V_{max} value of 15 μmol/mg/h and the affinity of 34 μM [32].

The properties of E-type ATPases have been intensively characterised in many tissues [8,33–37], including liver [8,12,13]. Surface ecto-ATPases and ecto-apyrases contain two predicted transmembrane domains at the N- and C-termini, share sequence homology with particular reference to 'apyrase conserved regions' and show immunological cross-reactivity [34–37]. The enzyme ecto-ATPase forms multimers [33] and is distributed in discrete clusters throughout the cell membrane [34]. In the liver, ecto-ATPase may serve at least two major functions: it terminates ATP/ADP-induced signal transduction and, in complex with ecto-5′-nucleotidase and nucleoside transporter, it participates in adenosine recycling [12,13].

4.2. Inhibitory effects of purinergic agents on rat liver ecto-ATPase: comparison with nucleotide-binding studies

We have recently reported steady-state analysis of [^{3}H]ATP interaction with binding sites on rat liver plasma membranes that were identified as ligand-binding components of P2Y receptors [17]. The displacement studies showed the following rank order of inhibitory potency for [^{3}H]ATP-binding sites: ATPγS > 2-MeSATP > ATP > ADPβS ≥ ADP ≫ RB2 ≫ suramin ≫ Ap_4A. The P2X agonists α,β-MeATP and β,γ-MeATP were much less potent inhibitors, whereas AMP, adenosine, Ap_5A, PPADS and non-adenine nucleoside triphosphates did not exert any inhibitory effects at concentration ranges of 10^{-5}–10^{-4}M. Some of these purinergic agents have been examined in the present work for inhibitory effects on liver ecto-ATPase, thus allowing a comparison of their effects on the kinetics of nucleotide-receptor interaction and on ATP hydrolysis in the same membrane preparations.

4.2.1. Non-hydrolysable P2 agonists

Ectoenzymatic breakdown of extracellular ATP and other nucleotide agonists always compromises the pharmacological classification of a given purinoceptor depending on the concentration and activity of the enzyme near the receptor [2,19]. Analogues of ATP with the non-bridging oxygen substituted by sulfur or with the bridging oxygen substituted with a CH_2 group are much less susceptible to degradation by ATPases [38] and are therefore widely used in pharmacological studies as potent purinergic agonists [4–6]. Phosphorothioates ATPγS and ADPβS (but not the methylene phosphonates α,β-MeATP and β,γ-MeATP) effectively compete with [^{3}H]ATP for nucleotide-binding sites on liver plasma membranes [17] and act as a full P2Y agonists in the liver [16]. However, we were unable to detect significant inhibition of liver ecto-ATPase activity in the presence of these non-hydrolysable nucleotides at concentration range of 10^{-3} M. Absence of any inhibitory effects of ATPγS have also been reported for ecto-ATPases of guinea pig urinary bladder [18], whereas in blood cells [23] and endothelial cells [24] this P2 agonist can concurrently act as ecto-ATPase inhibitor.

4.2.2. Diadenosine polyphosphates

Naturally occurring diadenosine polyphosphates are also relatively resistant to enzymatic hydrolysis (compared to ATP and other nucleotides) [2] and could act as messenger molecules through activation of P2 receptors in various tissues [6], including liver [39,40]. Although Ap_3A and Ap_4A are full glycogenolytic agonists in the liver, Ap_4A behaves more like ATP, whereas Ap_3A rather resembles ADP [40]. Likewise, only Ap_4A progressively inhibited both [^{3}H]ATP binding to liver plasma membranes [17] and its subsequent enzymatic hydrolysis (present work), whereas Ap_3A and Ap_5A did not exert any inhibitory effects on the measured parameters. The Ap_4A was also shown to be a weak ecto-ATPase inhibitor in vascular endothelial cells [7]. Although the presence of specific binding sites for Ap_4A and other polyphosphates on the cell surface cannot be ruled out, unambiguous association of physiological effects of these dinucleotides with a distinct class of receptors has not been achieved [6]. Our data suggest that modulatory effects of Ap_4A may be mediated (at least partially) by its competition with ATP for nucleotide-binding receptor sites and/or inhibition of ecto-ATPase activity.

4.2.3. P2 antagonists

Several antagonists with different selectivities for P2 receptor subtypes were tested for their ability to

affect liver ecto-ATPase, including suramin, RB2 and PPADS. All these compounds appear to be ecto-ATPase inhibitors, the first two being the most active and equipotent. Both suramin and RB2 inhibited [^{3}H]ATP hydrolysis at comparable concentration ranges (10^{-6}–10^{-4} M) to those in nucleotide-binding [17] and pharmacological [3–6] studies. Non-competitive ecto-ATPase inhibition by P2 antagonists suggest that their inhibitory effects are presumably caused by steric hindrance due to interaction with non-specific domains in the vicinity of ATP-binding sites. Another interesting explanation is that, since ecto-ATPase is modulated by intermolecular cross-linking [33], P2 antagonists can inhibit the enzyme activity by interfering with non-covalent monomer–monomer interactions and changes in the oligomerisation state.

4.3. Evaluation of structural/functional relationship between ecto-ATPases and P2 receptors

The integral membrane glycoproteins P2 receptors and ecto-ATPases represent two major classes of nucleotide-binding proteins on the cell surface that share similarities in ligand specificity. Both ecto-ATPases and P2 receptors are able to recognise a subset of nucleotides only if they contain di- or triphosphate chain with unmodified bridging oxygen. However, P2 receptors are predominantly activated by nucleotide uncomplexed to a metal cation (presumably ATP^{4-}), whereas the enzyme ecto-ATPase preferentially recognises the bidentate Mg^{2+}–nucleotide complex at its active site [41,42].

Based on the data from concurrent inhibition of ecto-ATPase activity and potentiation of responses to extracellular ATP in various tissues by P2 antagonists [18–22], a structural similarity between the nucleotide-binding sites of P2 receptors and the ATPase molecule has been proposed [20,22]. Although suramin and RB2 also competed with [^{3}H]ATP for nucleotide-binding sites [17] and progressively decreased ecto-ATPase activity (present work) in rat liver plasma membranes, such inhibition is presumably realised through unknown non-specific mechanisms rather than direct competition with ATP for nucleotide-binding sites. In any event, such inhibition of ecto-ATPase activity could mask true antagonistic potencies of P2-antagonists, thus limiting the usefulness of these compounds in studies of purinergic mechanisms.

Competitive data with various nucleotide agonists also indicate that, despite the apparent similarities, nucleotide-binding sites of P2 receptors and ecto-ATPases show clear-cut structural differences. Both adenine base and β- and γ-phosphate groups are involved in the initial step of nucleotide–receptor interaction [17], whereas conformation of the phosphate chain is presumably the most important motif responsible for subsequent ATP hydrolysis on liver surface. Such a suggestion is consistent with data which showed that E-type ATPases possess stereoselectivity towards the ribose moiety and polyphosphate chain (but not towards purine ring) [38] and contain the nucleoside phosphate binding domains which are likely to be more important in the hydrolysis rather than the binding of the substrate [37].

It should be taken into account that ATP-binding site is not a static structure and its conformation can be changed as the second substrate or cofactor is added [42]. Therefore, inhibitory effects of purinergic agents on the Mg^{2+}-dependent enzymatic ATP hydrolysis may differ significantly from that in nucleotide-binding studies performed after complete withdrawal of Mg^{2+} and other cations by chelating agent EDTA [17].

4.4. Effect of P2 antagonists and Ap_4A on the pattern of sequential ATP dephosphorylation

Another way in which the metabolism of ATP by tissues can lead to complications in purinoceptor analysis is the production of metabolites which are active as agonists in their own right with different, often opposite, effects [19]. Since inhibition of ecto-ATPase activity can modify the extent of phosphorylation of extracellular adenine nucleotides, we hypothesise that the whole pattern of nucleotide hydrolysis might also differ in the presence of certain purinergic agents. Sequential [^{3}H]ATP dephosphorylation still occurs in the presence of suramin, Ap_4A and PPADS, but the appearance of the ultimate reaction product, adenosine, was significantly delayed with time. It can be reasonably explained by a decrease in the rate of ATP hydrolysis with respective decrease of precursor substrate concentrations available for subsequent ADPase and AMPase reactions.

In contrast, in the presence of RB2 the hydrolysis of [^{3}H]ATP only follows the pattern ATP → ADP, since the formation of subsequent metabolites AMP and adenosine was virtually eliminated. The observed accumulation of ADP in the course of partial inhibition of ATP hydrolysis by RB2 suggests that hydrolysis of ATP and ADP on the liver surface is mediated by two separate ecto-enzymes. As such, the mechanism of RB2 action may involve complete inhibition of ADPase activity and/or blocking of ADP delivery to the enzyme in the course of inhibition of a preceding ecto-ATPase reaction.

In all cases, the inhibition of ecto-ATPase activity by purinergic agents is accompanied by profound changes in the whole sequence of nucleotide hydrolysis that can specifically regulate the physiological effects on cell metabolism of ATP and its dephosphorylated metabolites. The accumulated ATP and ADP can feed-forward inhibit 5′-nucleotidase activity [9] thus attenuating both adenosine production and activation of adenosine receptors. Whilst ATP and ADP are able to realise their effects through the same receptor sites on liver plasma membranes [17,43], the process of extracellular AMP recognition and binding presumably involve the membrane ecto-5′-nucleotidase which, along with catalytic function, can perform the role of a specific AMP receptor [43].

4.5. Concluding remarks

The studies were aimed at correlating the previously reported inhibitory effects of various purinergic agents on the kinetics of ATP-receptor interaction [17] and their subsequent enzymatic hydrolysis of ATP (present work). In this way, we have attempted to assess the structural/functional relationship between ecto-ATPases and P2 receptors on the cell surface. Only P2 antagonists and diadenosine tetraphosphate (Ap_4A) progressively and non-competitively inhibited Mg^{2+}-dependent enzymatic hydrolysis of [^{3}H]ATP in rat liver plasma membranes with the following rank order of inhibitory potency: suramin > RB2 ≫ Ap_4A > PPADS (Table 3). In addition, we have examined in detail the whole pattern of exogenous [^{3}H]ATP hydrolysis through stepwise reactions ATP → ADP → AMP → adenosine and have deduced that there are substantial differences in the observed hydrolysis sequence in the presence of ecto-ATPase inhibitors. Taken together, these data suggest that although nucleotide-binding sites of ecto-ATPase are distinct from that of P2 receptors, some purinergic agonists and antagonists can potentiate cellular responses to extracellular ATP through non-specific inhibition of the ensuing pathways of purine catabolism.

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References

[1] G.R. Dubyak, C. El-Moatassim, Signal transduction via P_2-purinergic receptors for extracellular ATP and other nucleotides, Am. J. Physiol. 265 (1993) C577–C606.
[2] H. Zimmermann, Biochemistry, localization and functional roles of ecto-nucleotidases in the nervous system, Prog. Neurobiol. 49 (1996) 589–618.
[3] G. Burnstock, The past, present and future of purine nucleotides as signalling molecules, Neuropharmacology 36 (1997) 1127–1139.
[4] M.P. Abbracchio, G. Burnstock, Purinoceptors: are there families of P2X and P2Y purinoceptors?, Pharmacol. Ther. 64 (1994) 445–475.
[5] B.B. Fredholm, M.P. Abbracchio, G. Burnstock, J.W. Daly, T.K. Harden, K.A. Jacobson, P. Leff, M. Williams, Nomenclature and classification of purinoceptors, Pharmacol. Rev. 46 (1994) 143–156.
[6] T.K. Harden, J.L. Boyer, R.A. Nicholas, P_2-purinergic receptors: subtype-associated signaling responses and structure, Annu. Rev. Pharmacol. Toxicol. 35 (1995) 547–579.
[7] J.D. Pearson, Ectonucleotidases: measurement of activities and use of inhibitors, in: D.M. Paton (Ed.), Methods in Pharmacology, Plenum Press, New York, 1985, pp. 83–105.
[8] L. Plesner, Ecto-ATPases: identities and functions, Int. Rev. Cytol. 158 (1995) 141–214.
[9] P. Meghji, J.D. Pearson, L.L. Slakey, Kinetics of extracellular ATP hydrolysis by microvascular endothelial cells from rat heart, Biochem. J. 308 (1995) 725–731.
[10] E.R. Lazarowski, L. Homolya, R.C. Boucher, T.K. Harden, Identification of an ecto-nucleoside diphosphokinase and its contribution to interconversion of P2 receptor agonists, J. Biol. Chem. 272 (1997) 20402–20407.
[11] B. Grobben, K. Anciaux, D. Roymans, C. Stefan, M. Bollen, E.L. Eshmans, H. Slegers, An ecto-nucleotide pyrophosphatase is one of the main enzymes involved in the extracellular metabolism of ATP in rat C6 glioma, J. Neurochem. 72 (1999) 826–834.

[12] M. Che, T. Nishida, Z. Gatmaitan, I.M. Arias, A nucleoside transporter is functionally linked to ectonucleotidases in rat liver canalicular membrane, J. Biol. Chem. 267 (1992) 9684–9688.
[13] M. Che, Z. Gatmaitan, I.M. Arias, Ectonucleotidases, purine nucleoside transporter, and function of the bile canalicular plasma membrane of the hepatocyte, FASEB J. 11 (1997) 101–108.
[14] S.F. Schlosser, A.D. Burgstahler, M.H. Nathanson, Isolated rat hepatocytes can signal to other hepatocytes and bile duct cells by release of nucleotides, Proc. Natl. Acad. Sci. USA 93 (1996) 9948–9953.
[15] R.M. Roman, Y. Wang, S.D. Lidofsky, A.P. Feranchak, N. Lomri, B.F. Scharschmidt, J.G. Fitz, Hepatocellular ATP-binding cassette protein expression enhances ATP release and autocrine regulation of cell volume, J. Biol. Chem. 272 (1997) 21970–21976.
[16] S. Keppens, The complex interaction of ATP and UTP with isolated hepatocytes. How many receptors?, Gen. Pharmacol. 24 (1993) 283–289.
[17] G.G. Yegutkin, G. Burnstock, Steady-state binding of [^{3}H]ATP to rat liver plasma membranes and competition by various purinergic agonists and antagonists, Biochim. Biophys. Acta 1373 (1998) 227–236.
[18] S.M.O. Hourani, J.A. Chown, The effects of some possible inhibitors of ectonucleotidases on the breakdown and pharmacological effects of ATP in the guinea-pig urinary bladder, Gen. Pharmacol. 20 (1989) 413–416.
[19] B.E. Crack, M.W. Beukers, K.C.W. McKechnie, A.P. Ijzerman, P. Leff, Pharmacological analysis of ecto-ATPase inhibition: evidence for combined enzyme inhibition and receptor antagonism in P_{2x}-purinoceptor ligands, Br. J. Pharmacol. 113 (1994) 1432–1438.
[20] A.U. Ziganshin, C.H.V. Hoyle, L.E. Ziganshina, G. Burnstock, Effects of cyclopiazonic acid on contractility and ecto-ATPase activity in guinea-pig urinary bladder and vas deferens, Br. J. Pharmacol. 113 (1994) 669–674.
[21] B.C. Chen, C.-M. Lee, W.-W. Lin, Inhibition of ecto-ATPase by PPADS, suramin and reactive blue in endothelial cells, C_6 glioma cells and RAW 264.7 macrophages, Br. J. Pharmacol. 119 (1996) 1628–1634.
[22] J.G. Stout, T.L. Kirley, Inhibition of purified chicken gizzard smooth muscle ecto-ATPase by P2 purinoceptor antagonists, Biochem. Mol. Biol. Int. 36 (1995) 927–934.
[23] M.W. Beukers, C.J.M. Kerkhof, A.M. Van Rhee, U. Ardanuy, C. Gurgel, H. Widjaja, P. Nickel, A.P. Ijzerman, W. Soudijn, Suramin analogs, divalent cations and ATPγS as inhibitors of ecto-ATPase, Naunyn Schmiedeberg's Arch. Pharmacol. 351 (1995) 523–528.
[24] B.C. Chen, W.-W. Lin, Inhibition of ecto-ATPase by the P_2 purinoceptor agonists, ATPγS, α,β-methylene-ATP, and AMP-PNP, in endothelial cells, Biochem. Biophys. Res. Commun. 233 (1997) 442–446.
[25] T.P. Kenakin, D. Beek, Self-cancellation of drug properties by a mode of organ selectivity: the antimuscarinic effects of ambenonium, J. Pharmacol. Exp. Ther. 232 (1985) 732–740.
[26] P.R. Dorling, R.N. Le Page, A rapid high yield method for the preparation of rat liver cell plasma membranes, Biochim. Biophys. Acta 318 (1973) 33–44.
[27] M.A.K. Markwell, S.M. Haas, L.L. Bieber, N.E. Tolbert, A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples, Analyt. Biochem. 87 (1978) 206–210.
[28] G.A. Norman, M.J. Follett, D.A. Hector, Quantitative thin-layer chromatography of ATP and the products of its degradation in meat tissue, J. Chromatogr. 90 (1974) 105–111.
[29] S.-H. Lin, Liver plasma membrane ecto-ATPase: purification, localization, cloning and functions, Ann. New York Acad. Sci. 603 (1990) 394–400.
[30] C. Stefan, W. Stalmans, M. Bollen, Growth-related expression of the ectonucleotide pyrophosphatase PC-1 in rat liver, Hepatology 28 (1998) 1497–1503.
[31] C. Stefan, R. Gijsberg, W. Stalmans, M. Bollen, Differential regulation of the expression of nucleotide pyrophosphatases/phosphodiesterases in rat liver, Biochim. Biophys. Acta 1450 (1999) 45–52.
[32] G.G. Yegutkin, S.M. Yakubovskii, G.G. Gatsko, Alteration of 5′-nucleotidase properties in rat adipose and liver plasma membranes after 1 Gy whole-body γ-irradiation, Int. J. Radiat. Biol. 63 (1993) 583–587.
[33] J.G. Stout, T.L. Kirley, Control of cell membrane ecto-ATPase by oligomerization state intermolecular cross-linking modulates ATPase activity, Biochemistry 35 (1996) 8289–8298.
[34] S. Lewis-Carl, T.L. Kirley, Immunolocalization of the ecto-ATPase and ecto-apyrase in chicken gizzard and stomach, J. Biol. Chem. 272 (1997) 23645–23652.
[35] B. Kegel, N. Braun, P. Heine, C.R. Maliszewski, H. Zimmermann, An ecto-ATPase and an ecto-ATP diphosphohydrolase are expressed in rat brain, Neuropharmacology 36 (1997) 1189–1200.
[36] J.S. Esch II, J. Sevigny, E. Kaczmarek, J.B. Siegel, M. Imai, K. Koziak, A.R. Beaudoin, S.C. Robson, Structural elements and limited proteolysis of CD39 influence ATP diphosphohydrolase activity, Biochemistry 38 (1999) 2248–2258.
[37] T.M. Smith, T.L. Kirley, Site-directed mutagenesis of a human brain ecto-apyrase evidence that the E-type ATPases are related to the actin/heat shock 70/sugar kinase superfamily, Biochemistry 38 (1999) 321–328.
[38] L.A. Welford, N.J. Cusack, S.M.O. Hourani, The structure–activity relationships of ectonucleotidases and of excitatory P2-purinoceptors: evidence that dephosphorylation of ATP analogues reduces pharmacological potency, Eur. J. Pharmacol. 141 (1987) 123–130.
[39] K.M. Craik, A.G. McLennan, M.J. Fisher, Adenine dinucleotide-mediated activation of glycogen phosphorylase in isolated liver cells, Cell. Signal. 5 (1993) 89–96.
[40] S. Keppens, Effects of diadenosine triphosphate and diadenosine tetraphosphate on rat liver cells. Differences and similarities with ADP and ATP, Biochem. Pharmacol. 52 (1996) 441–445.

[41] J. Pearson, N.J. Cusack, Investigation of the preferred Mg(II)-adenine-nucleotide complex at the active site of ecto-nucleotidases in intact vascular cells using phosphorothioate analogues of ADP and ATP, Eur. J. Biochem. 151 (1985) 373–375.
[42] M. Cohn, Structural and chemical properties of ATP and its metal complexes in solution, Ann. New York Acad. Sci. 603 (1990) 151–164.
[43] G.G. Yegutkin, G. Burnstock, Steady-state binding of adenine nucleotides ATP, ADP and AMP to rat liver and adipose plasma membranes, J. Rec. Sign. Transduc. Res. 19 (1999) 437–448.