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Steady-state binding of [^{3}H]ATP to rat liver plasma membranes and competition by various purinergic agonists and antagonists

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Abstract

Steady-state analysis of nucleotide-binding sites on rat liver plasma membranes was carried out using ^{3}H-labelled ATP as radioligand under complete inhibition of ecto-ATPase activity by excess EDTA. Binding of [^{3}H]ATP to the membranes is saturable, reversible and apparently involves one population of specific binding sites with K_d of about 90 nM and binding capacity (B_{max}) of 15 pmol/mg protein. A broad spectrum of purinergic agonists and antagonists was examined as potential inhibitors of the measured binding. The displacement studies showed the following rank order of inhibitory potency for [^{3}H]ATP-binding sites (pIC_{50} values in parentheses): ATPγS (7.49) > 2-MeSATP (7.18) > ATP (6.91) > ADPβS (6.64) ≥ ADP (6.56) ≫ RB2 (6.14) ≫ suramin (5.40) ≫ Ap_4A (4.57) > α,β-MeATP (4.19) ≥ β,γ-MeATP (3.97). AMP, adenosine, Ap_5A, PPADS, β-glycerophosphate as well as non-adenine nucleoside triphosphates GTP, UTP and CTP did not exert any effect on the measured binding at concentration ranges of 10^{-6}–10^{-4} M. In order to ascertain whether ATP and its analogues are capable of interacting with the same binding domain, 2-MeSATP and ADP were treated as alternative ligands that could compete with unlabelled ATP for its binding sites. A 2-fold increase of K_d value for ATP-receptor interaction was observed in the presence of 2-MeSATP (60 nM) or ADP (250 nM) without any modulation of B_{max} value, confirming that inhibitory effects of these compounds are competitive in nature. These studies demonstrate that ATP and its analogues are able to interact with a single binding domain on liver plasma membranes, which may be identified as ligand-binding component of P2 purinoceptors of the $P2Y_1$ subtype.

Keywords: Liver plasma membrane; P2Y purinoceptor; ATP binding; (Rat)

1. Introduction

Extracellular adenine nucleotides are widely accepted as effectors of signal transduction processes mediating signalling events in various tissues through P2 purinoceptors, whilst adenosine acts via P1 receptors [1–3]. This receptor classification was originally suggested by Burnstock [4] and then supplemented on the basis of agonist potency order and signal transduction mechanisms by the description of a number of different subtypes including P_{2X}, P_{2Y},

Abbreviations: ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); Ap_4A, diadenosine 5′,5‴-P^1,P^4-tetraphosphate; Ap_5A, diadenosine 5′,5‴-P^1,P^5-pentaphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); 2-MeSATP, 2-methylthio-ATP; α,β-MeATP, α,β-methylene ATP; β,γ-MeATP, β,γ-methylene ATP; EDTA, ethylenediaminetetraacetic acid (disodium salt); PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid; RB2, Reactive blue 2 (Cibacron blue 3GA); SDS, sodium dodecyl sulphate; TLC, thin-layer chromatography

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P_{2T}, P_{2Z}, P_{2U} and P_{2D} [5,6]. More recently, all of these subtypes have been categorized into two main families: a P2X family consisting of ligand-gated cation channels; and a P2Y family consisting of G protein-coupled receptors [3,7]. Although the pharmacology and physiology of P2 purinoceptors have been intensively investigated, studies of kinetic characteristics of these receptors are always complicated by rapid dephosphorylation of the exogenously applied or endogenously released ATP by plasma membrane ecto-ATPases [8,9]. Approaches to overcome this problem have involved the search for either non-hydrolysable P2 agonists and antagonists or appropriate compounds inhibiting membrane ecto-ATPase activity.

Since phosphonates containing α,β- or β,γ-methylene substituents have been shown to be relatively resistant to enzymatic hydrolysis and are considered the most active agonists for P2X receptors [5,6], [^{3}H]α,β-MeATP has been used in our laboratories to determine kinetic parameters and distribution of P2X receptors in bladder and vas deferens membranes [10–12]. Substitution of a phosphate group by a thiophosphate group also significantly increases resistance of the nucleotide to the ecto-nucleotidase activity, therefore [^{35}S]ATPγS [13,14] and [^{35}S]ADPβS [15,16] have been used as potential radioligands for P2Y purinoceptors. Although a number of reports have suggested successful labelling of P2 purinoceptors [6,10–18], comprehensive comparison of these data is limited by lack of a uniform approach and diversity of radioligands used.

For estimation of 'true' kinetic mechanisms of ATP-receptor interaction on the cellular surface it would be useful if the ecto-ATPases had a specific inhibitor of their own. Several compounds inhibiting ecto-ATPase activity have been examined as potentially valuable probes in P2 receptor studies [9,19–22] although none were found to be selective. Moreover, the possibility exists that ecto-ATPase and P2 receptors have very similar nucleotide-binding domains [20,23], therefore simple competitive displacement of the ligand and a reduction of its concentration in the vicinity of the receptor in the presence of selective ATPase inhibitor cannot be excluded. From this viewpoint, non-specific inhibition of the enzymes may be considered as a more preferable tool. Such compounds as paraformaldehyde [24], glycerol [25,26] and EDTA [14,16,27] were used for determination of nucleotide-binding parameters under partial or complete inhibition of certain ecto-nucleotidase activity.

In the present work, the chelating agent EDTA was examined as suitable non-specific inhibitor of ecto-ATPase activity to aid kinetic and competitive analysis of nucleotide-binding sites on rat liver plasma membranes using [^{3}H]ATP as radioligand and a broad spectrum of P2 receptor agonists and antagonists.

2. Materials and methods

2.1. Materials and equipment

2-MeSATP was from Research Biochemicals, PPADS was from Tocris Cookson Chemical. [2,8-^{3}H]ATP with specific activity 40 Ci/mmole was purchased from ICN Biomedicals (Belgium). Organic solvents were from Analar. Glass microfibre filters GF/B were from Whatman (UK). Liquid scintillation cocktail Wallac OptiPhase 'HiSafe'-3 was from Fisher Chemical. TLC plates were 20×10 cm silica gel 60 F_{254} type supplied by Sigma-Aldrich Chemicals. All other chemicals were purchased from Sigma Chemicals (Poole, UK).

The following Beckman equipment was used throughout the present studies: DU-65 spectrophotometer with programming QUANT-II Soft-Pack Module; J2-MC centrifuge with JA-20 rotor and Optima L-70 preparative ultracentrifuge with SW-41 bucket rotor; LS6000IC liquid scintillation spectrometer.

2.2. Liver plasma membrane isolation

Male Sprague-Dawley rats weighing 200–220 g were killed by asphyxiation with CO_2. The liver was perfused with saline through the portal vein for blood clearance, removed from the animal and placed in an ice-cold 1 mM borate buffer, containing 0.5 mM $CaCl_2$ (pH 7.5). About 5 g of the tissue was cut into small pieces, passed through 0.5 mm press-sieve and dispersed in glass homogenizer with teflon pestle. Further separation of plasma membrane frac-

tions from the bulk of contaminating mitochondria and microsomes was performed by a series of differential and isopycnic centrifugations as described previously [28]. The resulting membrane pellet was suspended in 7 mM Tris-HCl (pH 7.4) at 2–2.5 mg protein/ml and stored in aliquots in liquid nitrogen. Aliquots were used within 1 month following the preparation, thawed on a water bath at 37°C just before the experiment.

After first centrifuging of the crude liver homogenate at $150\times g$ for 10 min and discarding of the nuclear pellet, aliquots of the supernatant homogenate were also sampled and stored in liquid nitrogen until used in comparative enzyme and binding assays.

2.3. Measurement of [^{3}H]ATP binding to membranes

Plasma membranes (30–35 μg protein) were incubated for 5 min at 37°C in a final volume of 200 μl in the medium containing 50 mM Tris-HCl (pH 7.4), 30 mM EDTA, [^{3}H]ATP (approx. 10^6 dpm), unlabelled ATP (0–1000 nM) and various concentrations of other agents (in the case of competitive studies). The reaction was terminated by addition of 4 ml phosphate buffer (5 mM, pH 7.4) followed by rapid filtration through GF/B filters and subsequent 2-fold washing with phosphate buffer. The filters were staged overnight with 5 ml of scintillation cocktail and counted on a β-spectrometer. Specific binding was determined as the difference between total and non-specific binding (measured in the presence of 10 μM ATP) and it was no less than 85% of the total binding.

2.4. Enzyme assays

TLC assay has proved to be one of the most convenient and versatile methods for screening the adenine nucleotide catabolism [8], and therefore this approach was used both for determination of specific activity of ecto-ATPase (EC 3.6.1.15) and for estimation of the extent of [^{3}H]ATP degradation in the course of its binding to membranes. For ecto-ATPase assay the specimens were incubated for 10–15 min at 37°C in a final volume of 50 μl containing either 50 μg of homogenate or 5–7 μg of plasma membrane protein, 1 mM ATP with tracer [^{3}H]ATP (5×10^5 dpm), 100 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 200 μM ouabain and 5 mM NaN_3. To characterize the extent of [^{3}H]ATP degradation, the studies were conducted either as described for the binding assays (see Section 2.3) or with 5 mM $MgCl_2$ instead of 30 mM EDTA. In both cases the reaction was terminated by applying aliquots of the mixture (6 μl) on TLC sheets following the application of standard solution containing 2 mM ATP, ADP, AMP and adenosine on the same spot. ATP and its derivatives were separated by TLC with isobutyl alcohol/isoamyl alcohol/2-ethoxyethanol/ammonia/H_2O (9:6:18:9:15) as solvent [29] and visualized in UV light. The spots corresponding to ATP were scraped into scintillation vials followed by 1 h extraction of the nucleotide from the silica using 0.8 ml 0.1 N HCl. The residual [^{3}H]ATP was quantified by scintillation counting after an overnight incubation with 5 ml scintillation cocktail.

5′-Nucleotidase activity (EC 3.6.1.5) was assayed as previously described [26] and inorganic phosphate liberated after AMP hydrolysis was detected turbidimetrically [30].

2.5. Protein assay

Protein was determined by a modified Lowry method [31] using bovine serum albumin as standard after pre-incubation of the membrane samples with 1% SDS at 37°C for 45 min.

2.6. Data analysis

The maximum binding capacity (B_{max}) and the dissociation constant (K_d) for ATP-binding sites were determined using EBDA-LIGAND software (McPherson BioSoft, Cambridge, UK). Data from competition-binding experiments were subjected to computer analysis by non-linear least-squares curve fitting (GraphPad Prism software) to determine IC_{50} values. Objective statistical criteria (*F*-test, extra sum of squares principle) were used to evaluate goodness of fit and for discriminating between one-site and two-site binding models.

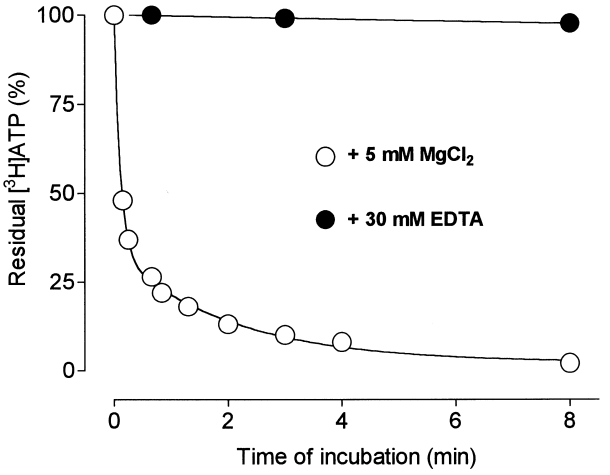

Fig. 1. Enzymatic hydrolysis of [^{3}H]ATP by rat liver plasma membranes. The membranes (30 μg protein) were incubated at 37°C with 50 nM [^{3}H]ATP in 50 mM Tris buffer (pH 7.4) containing either 30 mM EDTA or 5 mM $MgCl_2$ in the starting volume of 0.2 ml. Aliquots of the reaction mixture (6 μl) were periodically applied on TLC sheets for separation of the [^{3}H]ATP and its derivatives (Section 2.4). The residual [^{3}H]ATP after ATPase reaction is expressed on the ordinate as percentage of the initial concentration. The graphs show mean data ($n=3$); the standard error of the mean did not exceed the size of symbols.

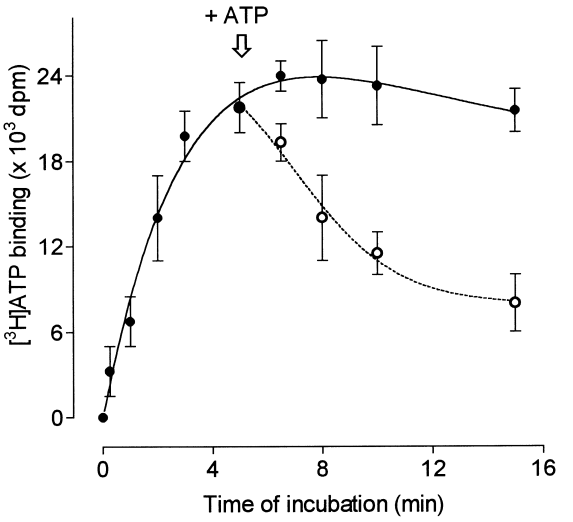

Fig. 2. Association-dissociation pattern for specific [^{3}H]ATP binding to rat liver plasma membranes. The association curve was obtained by incubation of the membranes with [^{3}H]ATP (50 nM) at 37°C in 50 mM Tris buffer supplemented with 30 mM EDTA for the indicated times. Following [^{3}H]ATP, excess ATP (10 μM) was added to the assay buffer 5 min later (indicated by arrow) and the time course of the membrane-bound [^{3}H]ATP dissociation is also shown by the dotted line. The ordinate shows the value of specific [^{3}H]ATP binding determined by subtracting non-specific binding from total binding figures. Data represent means ± S.E.M. of three experiments performed in duplicate.

3. Results

3.1. Determination of equilibrium conditions for [^{3}H]ATP binding to membranes

As shown in Fig. 1, rapid enzymatic dephosphorylation of [^{3}H]ATP (50 nM) was observed within the first minute after incubation with liver membranes, so that all attempts to detect any amount of the membrane-bound [^{3}H]ATP by binding assay showed radioactivity of the filters comparable with blank values. In the presence of 30 mM EDTA complete inhibition of ATPase activity was achieved (Fig. 1) and binding of [^{3}H]ATP to plasma membranes in these conditions reached a plateau value after 3–4 min and remained unchanged for at least 10 min (Fig. 2). When ^{3}H-labelled and unlabelled ATP (10 μM) were introduced to the assay buffer separately at 5 min interval, a gradual decrease of [^{3}H]ATP binding ensued (Fig. 2), indicating the reversible charac-

Table 1
Comparison of 5′-nucleotidase and ecto-ATPase specific activities and [^{3}H]ATP-binding capacity in rat liver homogenate and plasma membrane preparations

Preparation	5′-Nucleotidase (μmol P_i/mg/h)	Ecto-ATPase (μmol ATP/mg/h)	[^{3}H]ATP binding (dpm/mg ($\times 10^3$))
Homogenate	1.06 ± 0.13 (5)	1.52 ± 0.21 (3)	60.5 ± 11.4 (3)
Plasma membranes	13.80 ± 0.22 (8)	18.24 ± 0.52 (6)	545.0 ± 58.0 (6)

Ecto-ATPase and 5′-nucleotidase assays were carried out as described in Section 2.4 using either 50 μg of liver crude homogenate or 5–7 μg of purified plasma membranes and expressed as μmole of substrate hydrolysed by mg protein per hour. [^{3}H]ATP-binding assays were performed as described in Section 2.3 either with liver homogenate (50–250 μg/sample) or plasma membranes (10–45 μg/sample) (see Fig. 3) and the relative binding capacity was expressed as the amount of specific radioactivity bound per mg protein. Values are the mean ± S.E.M. with the number of experiments shown in parentheses.

ter of the nucleotide-binding process in the presence of EDTA.

The quantity of [^{3}H]ATP bound to plasma membranes increased proportionally with protein concentration over the range 10–45 μg (Fig. 3) and the relative amount of radioactivity bound per mg of membrane protein was about 9-fold higher than that for the crude homogenate containing substantial amounts of non-plasma membrane material (Table 1). Comparison of this value with the 12-fold extent of plasma membrane enrichment, as evaluated by enzyme markers 5′-nucleotidase and ecto-ATPase (Table 1), testifies that the measured ATP binding is predominately associated with plasma membranes rather than with other subcellular fractions. No [^{3}H]ATP binding was observed after preliminary treatment of the membranes with α-chymotrypsin (5 μg/sample; 10 min at 37°C), suggesting that nucleotide interaction with plasma membranes occurred at specific protein domains.

3.2. Kinetic analysis of [^{3}H]ATP interaction with plasma membranes

Steady-state analysis of ATP-receptor interaction was performed by adding [^{3}H]ATP and increasing concentrations of unlabelled ATP until saturation was achieved (Fig. 4). Graphical representation of this binding isotherm in a Scatchard plot showed linear dependence (Fig. 5). The kinetic constants (K_d and B_{max}) were determined by computer analysis using a model of single binding site with non-specific binding and are summarized in Table 2.

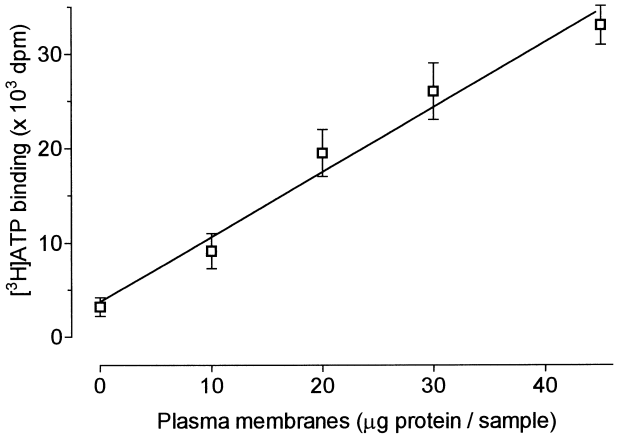

Fig. 3. Linearity of [^{3}H]ATP binding to rat liver plasma membranes with protein concentration. Increasing concentrations of plasma membrane protein (0–45 μg) were incubated for 5 min at 37°C in the final volume of 0.2 ml, containing 50 nM [^{3}H]ATP, 50 mM Tris (pH 7.4) and 30 mM EDTA. The ordinate shows the value of total [^{3}H]ATP binding. Data represent means ± S.E.M. of three experiments performed in duplicate.

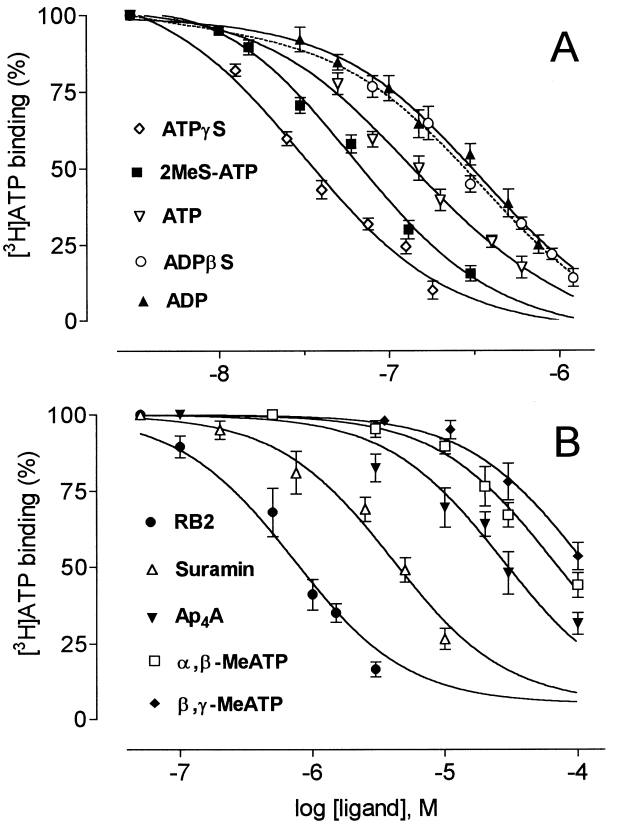

Fig. 4. Competitive inhibition of [^{3}H]ATP binding by purinergic ligands. The membranes were incubated with [^{3}H]ATP (50 nM) and increasing concentrations of unlabelled agents. The results are plotted as the percentage of maximal [^{3}H]ATP binding, measured in the absence of competitors. Means ± S.E.M. from three experiments performed in duplicate.

It should be noted that the amount of [^{3}H]ATP bound to membranes in the presence of excess ATP (10 μM) did not significantly exceed the blank values determined in the absence of plasma membrane protein. These data indicate that the detected non-specific binding of [^{3}H]ATP is primarily conditioned by partial adsorption of radioligand on the GF/B filters during filtration procedure, and not by non-specific interaction with any membrane proteins or low-affinity binding to another class of ATP receptors on liver plasma membranes.

Table 2
Kinetic parameters of [^{3}H]ATP binding to rat liver plasma membranes and their modification in the presence of 2-MeSATP and ADP as alternative ligands

Alternative ligand	Concentration of the ligand (nM)	ATP-binding parameters	
		B_{max} (pmol/mg protein)	K_d (nM)
None (control)	–	14.96 ± 0.85	88.2 ± 9.4
ADP	250	16.37 ± 1.21	180.9 ± 20.0*
2-MeSATP	60	15.8 ± 1.80	227.8 ± 31.5*

For determination of nucleotide-binding parameters plasma membranes were incubated with [^{3}H]ATP and increasing concentrations of the unlabelled ATP in the absence (control) or presence of the fixed concentration of certain inhibitor. The number of nucleotide-binding sites (B_{max}) and dissociation constant (K_d) were calculated from the corresponding Scatchard plots (see Fig. 5) by a computer-adapted least-squares method. Values are the mean ± S.E.M. of three to six experiments. Student's *t*-test was employed to compare the difference between means. *$P < 0.05$ as compared with control.

3.3. Competitive analysis of [^{3}H]ATP binding to plasma membranes

Addition of various P2Y agonists (Fig. 4A) and the P2 antagonists RB2 and suramin (Fig. 4B) to the assay buffer was accompanied by concentration-dependent inhibition of [^{3}H]ATP binding to membranes indicating the measurement of the radioligand binding to P2 receptors by this protocol. The diadenine nucleotide Ap_4A and the P2X agonists α,β-MeATP and β,γ-MeATP were much less potent competitors (Fig. 4B) whereas AMP, adenosine, Ap_5A, PPADS, β-glycerophosphate, GTP, UTP and CTP did not exert any inhibitory effect at concentration ranges of 10^{-6}–10^{-4} M (data not shown).

Table 3
Competitive analysis of [^{3}H]ATP binding to rat liver plasma membranes

Ligand	pIC_{50}
ATPγS	7.492 ± 0.043
2-MeSATP	7.181 ± 0.044
ATP	6.915 ± 0.027
ADPβS	6.640 ± 0.031
ADP	6.565 ± 0.028
RB2	6.143 ± 0.049
Suramin	5.396 ± 0.044
Ap_4A	4.569 ± 0.053
α,β-MeATP	4.195 ± 0.037
β,γ-MeATP	3.970 ± 0.044

The binding of 50 nM [^{3}H]ATP to liver membranes was inhibited by increasing concentrations of the indicated ligand. Binding competition curves were constructed (see Fig. 4) and fitted to a one-site model using non-linear least-squares curve fitting in GraphPad Prism. The constants are expressed in terms of pIC_{50} ($-\log IC_{50}$) ± S.E.M. for three independent experiments.

The pIC_{50} values were calculated from competition binding curves using one-site model and are summarized in Table 3. Overall, the displacement studies showed that the rank order of inhibitory potency for [^{3}H]ATP-binding sites was ATPγS > 2-MeSATP > ATP > ADPβS ≥ ADP ≫ RB2 ≫ suramin ≫ Ap_4A > α,β-MeATP ≥ β,γ-MeATP.

In order to check whether ATP and its analogues interact with the same binding domain, the most commonly used P2Y agonists 2-MeSATP and ADP [5–7] were treated as alternative ligands that could compete with unlabelled ATP for its binding sites.

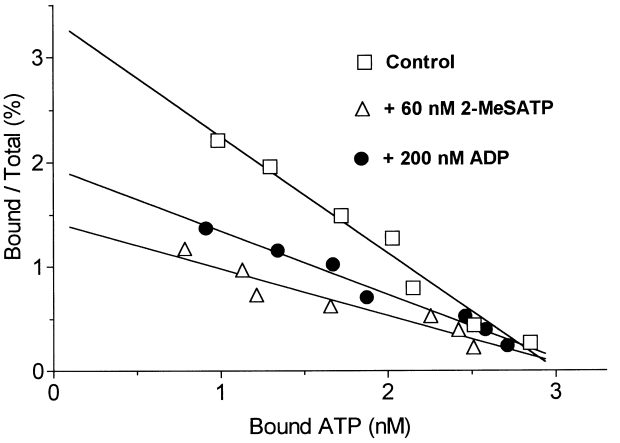

Fig. 5. Scatchard analysis of [^{3}H]ATP binding to liver plasma membranes in the presence of 2-MeSATP and ADP as alternative ligands. The membranes were incubated with 50 nM [^{3}H]ATP and unlabelled ATP (0–1000 nM) in the absence (control) or presence of 2-MeSATP (60 nM) or ADP (250 nM). Data are plotted as specifically bound radioligand (abscissa) versus bound ligand divided by total ligand concentration (ordinate). The data are the mean of duplicate assays from a representative experiment, which was repeated at least three times.

In the presence of fixed concentrations of 2-MeSATP (60 nM) or ADP (250 nM) saturation curves for [^{3}H]ATP binding to liver membranes were shifted to the right as the concentration of unlabelled ATP was increased. Scatchard transformation of these data is presented in Fig. 5. Computer analysis of the binding isotherms revealed a 2-fold increase of K_d value in the presence of 2-MeSATP and ADP without any modification of binding capacity, B_{max} (Table 2), confirming that inhibitory effects of these ATP analogues are competitive in nature.

4. Discussion

4.1. Kinetic and competitive analysis of ATP binding to plasma membranes

Understanding the dynamic biochemistry of P2 receptors requires development of truly reliable binding assays for their direct quantitation, and the data presented above suggest that the chelating agent EDTA can be readily used for this purpose. Addition of excess EDTA to the assay buffer was accompanied by complete inhibition of ecto-ATPase activity with respective stabilization of the nucleotide-binding process and in this way, steady-state binding of native [^{3}H]ATP to rat liver plasma membranes was carried out. Binding of ATP to the membranes is saturable, reversible and apparently involves one population of nucleotide-binding sites with K_d of about 90 nM and B_{max} of 15 pmol/mg protein.

Various nucleotide analogues were examined as potential inhibitors of the measured [^{3}H]ATP binding, including P2Y agonists, 2-MeSATP, ATPγS, ADPβS and ADP [5–7,32–34]; P2X agonists, α,β-MeATP and β,γ-MeATP [5–7]; diadenosine polyphosphates, Ap_4A and Ap_5A, which act as physiologically significant purinergic agonists at both P2X and P2Y receptors [22,35,36]. P2Y agonists were in equimolar competition with [^{3}H]ATP for its binding sites with the following rank order of inhibitory potency: ATPγS > 2-MeSATP > ATP > ADPβS ≥ ADP. Moreover, the affinity of ATP-binding sites was markedly reduced in the presence of 2-MeSATP and ADP as alternative ligands, indicating that displacement effects of these ATP analogues are caused by true competition with ATP for the common binding domain, and not by any allosteric constraints in the course of their interaction with other binding sites in the vicinity of ATP receptors. α,β-MeATP, β,γ-MeATP and Ap_4A were less potent competitors, whereas Ap_5A, AMP, adenosine and non-adenine nucleoside triphosphates did not cause any displacement. It may be concluded that both the adenine ring and an unmodified phosphate chain with two or three phosphate groups are involved in the nucleotide interaction with these binding sites.

Several P2 antagonists with different selectivities for P2 receptor subtypes were also examined in our binding studies. The trypanocide suramin and the dye RB2, which have been widely employed as P2Y antagonists [5,21,23], were capable of inhibiting [^{3}H]ATP binding to plasma membranes at a comparable concentration range (10^{-6}–10^{-5} M) as in pharmacological studies, suggesting that antagonistic effects of these compounds may be explained by direct competition with ATP for receptor sites. With another compound tested, PPADS, a putative antagonist of both P2Y and P2X receptors in various tissues [5,21,22], we were unable to detect any inhibitory effects even at concentration up to 50 μM.

4.2. Comparison with other liver radioligand-binding assays

ATP-binding sites were initially described in rat liver plasma membranes and hepatocytes using [^{35}S]ATPαS as radioligand [17] and reported later in human liver plasma membranes and in rabbit and guinea pig hepatocytes (see [18]). Although [^{35}S]ATPαS-binding studies with rat liver membranes also revealed one binding component with K_d 230 nM and B_{max} 30 pmol/mg protein [17], the most potent P2Y agonist 2-MeSATP possessed little affinity for these receptor sites [34]; in addition, a 'non-saturable' binding component of unknown physiological significance was detected. A disadvantage of ATPαS is its liability to enzymatic breakdown, so that steady-state conditions may be achieved only within the first minute of incubation.

Despite binding studies with the more stable [^{35}S]ATPγS having been performed on rat liver plasma membranes (IC_{50} for ATP, 600 nM) [13], identification of these receptors was limited by progressive non-specific binding of the radioligand with other

membrane proteins. Another non-hydrolysable radioligand [^{35}S]ADPβS has been used for labelling of P2Y receptors on rat liver parenchymal cells [16]. However, determination of the K_d value in these binding studies was precluded by high K_i value for unlabelled ADPβS (1.8 μM). Moreover, displacement data obtained with these non-hydrolysable ATP analogues do not conform to the affinity profile of known P2 receptors [6,13,14], which may be explained by differences in catabolism of the radioligand and unlabelled agonists.

Use of EDTA for complete inhibition of enzymatic breakdown of ATP and its analogues confers certain advantages compared with other binding assays. First, kinetic parameters of nucleotide-receptor interaction may be performed with native ATP as a 'kindred' ligand of P2 receptors and second, nucleotide-binding sites may be subdivided on the basis of relative order of potency of native ATP displacement by a series of purinergic agonists and antagonists.

4.3. Evaluation of physiological relevance of the described binding sites

Although the system described provides a useful model for steady-state analysis of nucleotide-receptor interaction on the cellular surface, it is not possible to perform signal transduction processes under similar conditions. Therefore, comparison of the binding constants with receptor activity constants may be considered a useful tool to establish whether these binding sites constitute a physiologically relevant receptor.

The most prominently described second messenger response to $P2Y_1$ receptor activation in liver is activation of the rate-limiting enzyme of glycogenolysis glycogen phosphorylase and mobilization of intracellular calcium which was originally described by Charest et al. [32]. Summarizing the literature data concerning activation of glycogen phosphorylase in isolated hepatocytes by various P2Y agonists [32–36] reveals the following rank order of potency: 2-MeSATP > ADP ≥ ATP > Ap_3A > Ap_4A. Other responses mediated by ATP and its analogues in liver include accumulation of cyclic AMP, K^+ uptake, activation of phospholipases C and D, release of eicosanoids, phospholipid base exchange, etc. [1,13,18,33,37]. In contrast to glycogenolytic effects (detected within the first 10–20 s), these responses were assayed after longer incubation period and 100–1000-fold higher ATP concentrations were required to induce effects.

There is a good correlation between ability of ATP analogues to induce glycogenolytic effects in isolated hepatocytes and their relative potency for [^{3}H]ATP displacement in our studies with isolated liver plasma membranes. Moreover, both effects were observed over the comparable concentration range suggesting that identical sites have been labelled. On the basis of this correlation it may be speculated that the described nucleotide-binding sites represent a specific binding component of P2 receptors (presumably of $P2Y_1$ subtype), directly participating in the purinergic action of ATP and ADP on liver metabolism. Some features of the single binding sites that we have revealed are not consistent with the hypothesis that multiple receptors for ATP exist in liver [18]. These apparent discrepancies may be due to linkage of a single nucleotide-binding domain to multiple second messenger signalling pathways. Alternatively, the possibility that at higher concentrations (10^{-5}–10^{-4} M) ATP can mediate certain responses through other 'non-purinergic' receptor sites cannot be excluded.

4.4. Assessment of EDTA suitability for nucleotide-binding studies

Another question concerning the reliability of the proposed procedure is the presence of EDTA in the assay buffer and the absence of Mg^{2+} and other cations. Cooper et al. [15] noticed that in the presence of $MgCl_2$ the binding of [^{35}S]ADPβS to P2 receptors on turkey plasma membranes was not readily saturable, whereas omission of Mg^{2+} revealed a saturable high affinity binding site and significantly increased radioligand binding to membranes. Similar results were obtained more recently in kinetic experiments on [^{3}H]αβ-MeATP binding to P2X receptors in human urinary bladder [11], where withdrawal of Mg^{2+} from the incubation medium drastically increased the nucleotide-binding capacity of the membrane preparations. Binding of various labelled ATP analogues to high-affinity binding sites was increased in the presence of 1 mM EDTA and, what is more, this chelating agent prevented interaction of the ligands

with non-specific binding sites and significantly reduced their enzymatic breakdown [14,16]. The foregoing data indicate that P2 receptors are activated by a form of ATP uncomplexed to divalent cations (presumably ATP^{4-}) and not by ATP-Mg^{2+}, which predominates in biological fluids and constitutes the substrate of ATPases and kinases [38,39]. As such, it may be assumed that complete withdrawal of the residual Mg^{2+} by EDTA will not be accompanied by any significant modulation of nucleotide-binding parameters.

4.5. Concluding remarks

In summary, these studies (a) provide accurate parameters for the binding of native [^{3}H]ATP to cellular surface in the absence of complications arising from the further metabolism of the radioligand; (b) establish the relative order of potency of [^{3}H]ATP displacement by a broad spectrum of purinergic agents; (c) demonstrate that both ATP and its analogues 2-MeSATP and ADP are able to interact with a common binding domain on liver plasma membranes; (d) suggest that the described binding sites are linked to $P2Y_1$ receptors, mediating, in particular, ATP/ADP-induced activation of liver glycogen-phosphorylase and accumulation of intracellular Ca^{2+}.

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References

[1] C. El-Moatassum, J. Dornard, J.-C. Mani, Extracellular ATP and cell signalling, Biochim. Biophys. Acta 1134 (1992) 31–45.
[2] G. Burnstock, P2 purinoceptors: historical perspective and classification, in: D.J. Chadwick, J.A. Goode (Eds.), P2 Purinoceptors: Localization, Function and Transduction Mechanisms, Ciba Found. Symp. 198, Wiley, Chichester, 1996, pp. 1–34.
[3] G. Burnstock, The past, present and future of purine nucleotides as signalling molecules, Neuropharmacology 36 (1997) 1127–1139.
[4] G. Burnstock, A basis for distinguishing two types of purinergic receptors, in: R.W. Straub, L. Bolis (Eds.), Cell Membrane Receptors for Drugs and Hormones, a Multidisciplinary Approach, Raven Press, New York, 1978, pp. 107–118.
[5] B.B. Fredholm, M.P. Abbracchio, G. Burnstock, J.W. Daly, T.H. 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] M.P. Abbracchio, G. Burnstock, Purinoreceptors: are there families of P2X and P2Y purinoceptors?, Pharmacol. Ther. 64 (1994) 445–475.
[8] 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.
[9] H. Zimmermann, Biochemistry, localization and functional roles of ecto-nucleotidases in the nervous system, Prog. Neurobiol. 49 (1996) 589–618.
[10] X. Bo, G. Burnstock, Triphosphate, the key structure of the ATP molecule responsible for interaction with P_{2x}-purinoceptors, Gen. Pharmacol. 24 (1993) 637–640.
[11] X. Bo, G. Burnstock, Characterization and autoradiographic localization of [^{3}H]α,β-methylene adenosine 5′-triphosphate binding sites in human urinary bladder, Br. J. Urol. 76 (1995) 297–302.
[12] M. Zhao, X. Bo, C.F. Neely, G. Burnstock, Characterization and autoradiographic localization of [^{3}H]α,β-methylene ATP binding sites in cat urinary bladder, Gen. Pharmacol. 27 (1996) 509–512.
[13] K.C. Malcolm, S.E. Trammell, J.H. Exton, Purinergic agonist and G protein stimulation of phospholipase D in rat liver plasma membranes. Independence from phospholipase C activation, Biochim. Biophys. Acta 1268 (1995) 152–158.
[14] S. Motte, S. Swillens, J.-M. Boeynaems, Evidence that most high-affinity ATP binding sites on aortic endothelial cells and membranes do not correspond to P_2 receptors, Eur. J. Pharmacol. 307 (1996) 201–209.
[15] C.L. Cooper, A.J. Morris, T.K. Harden, Guanine nucleotide-sensitive interaction of a radiolabeled agonist with a phospholipase C-linked P_{2y}-purinergic receptor, J. Biol. Chem. 264 (1989) 6202–6206.
[16] A.M. van Rhee, E.C.A. van Winden, J.F. Nagelkerke, H.J.G.M. De Bont, A.P. IJzerman, W. Soudijn, Binding of the radioligand [^{35}S]adenosine 5′-*O*-(2-thiodiphosphate) and intracellular calcium response in rat liver parenchymal cells, Biochem. Pharmacol. 45 (1993) 801–807.
[17] S. Keppens, H. De Wulf, Characterization of the liver P_{2-}purinoceptor involved in the activation of glycogen phosphorylase, Biochem. J. 240 (1986) 367–371.
[18] S. Keppens, The complex interaction of ATP and UTP with isolated hepatocytes. How many receptors?, Gen. Pharmacol. 24 (1993) 283–289.
[19] S.M.O. Hourani, J.A. Chown, The effects of some possible inhibitors of ectonucleotidases on the breakdown and phar-

macological effects of ATP in the guinea-pig urinary bladder, Gen. Pharmacol. 20 (1989) 413–416.
[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] T.D. Westfall, C.A. McIntyre, S. Obeid, J. Bowes, C. Kennedy, P. Sheddon, The interaction of diadenosine polyphosphates with P2X-receptors in the guinea-pig isolated vas deferens, Br. J. Pharmacol. 121 (1997) 57–62.
[23] 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.
[24] J.R. Jefferson, J.T. Harmon, G.A. Jamieson, Identification of high-affinity (K_d 0.35 μmol/L) and low-affinity (K_d 7.9 μmol/L) platelet binding sites for ADP and competition by ADP analogues, Blood 71 (1988) 110–116.
[25] S.M. Yakubovskii, G.N. Fil'chenkov, V.K. Naumenko, T.G. Lastovskaya, G.G. Gatsko, Interaction of [^{3}H]AMP with liver cells and their plasma membranes, Biochemistry (Moscow) (Engl. transl.) 56 (1991) 369–373.
[26] 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.
[27] G.G. Yegutkin, Interaction of native 5′-ATP with plasma membranes of rat liver and adipose tissue, Biochemistry (Moscow) (Engl. transl.) 59 (1994) 1109–1112.
[28] 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.
[29] 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.
[30] H. Eibl, W.E.M. Lands, A new sensitive determination of phosphate, Anal. Biochem. 30 (1969) 51–57.
[31] 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, Anal. Biochem. 87 (1978) 206–210.
[32] R. Charest, P.F. Blackmore, J.H. Exton, Characterization of responses of isolated rat hepatocytes to ATP and ADP, J. Biol. Chem. 260 (1985) 15789–15794.
[33] S. Keppens, H. De Wulf, Some P_2 purinergic agonists increase cytosolic calcium but not inositol 1,4,5-triphosphate in isolated rat hepatocytes, Biochim. Biophys. Acta 1269 (1995) 316–322.
[34] S. Keppens, H. De Wulf, Characterization of the biological effects of 2-methylthio-ATP on rat hepatocytes: clear-cut differences with ATP, Br. J. Pharmacol. 104 (1991) 301–304.
[35] 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.
[36] 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.
[37] D. Häussinger, Regulation of hepatic metabolism by extracellular nucleotides and eicosanoids, J. Hepatol. 8 (1989) 259–266.
[38] M. Cohn, Structural and chemical properties of ATP and its metal complexes in solution, Ann. NY Acad. Sci. 603 (1990) 151–164.
[39] 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.