Characterization of the binding of diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) to rat liver cell membranes

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Diadenosine polyphosphates present in the extracellular environment can, through interaction with appropriate purinoceptors, influence a range of cellular activities. Here we have investigated the nature of the ligand:receptor interactions involved in diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A)-mediated stimulation of glycogen breakdown in isolated rat liver cells. [2-^{3}H]Ap_4A showed specific binding to both intact isolated liver cells and plasma membrane fractions prepared from isolated liver cells. HPLC analysis confirmed that binding was mediated by intact Ap_4A and not by potential breakdown products (e.g. ATP, adenosine etc). Binding of [2-^{3}H]Ap_4A, to isolated liver cell plasma membrane preparations, was successfully displaced by a range of both naturally occurring and synthetic diadenosine polyphosphates with the rank order potency $Ap_4A \geqslant Ap_5A > Ap_6A > Ap_3A > Ap_2A$. [2-^{3}H]$Ap_4A$ binding was not displaced by P_1 effectors but was successfully displaced by a range of P_2 effectors with the rank order potency 2-methylthio-ATP > ATP > ADP ⩾ adenosine 5′-[$\alpha\beta$-methylene]triphosphate > adenosine 5′-[$\beta\gamma$-methylene]triphosphate. These findings are consistent with the interaction of Ap_4A with a P_{2y}-like subclass of purinoceptor and are discussed in relation to (1) the known purinoceptor populations in liver cell plasma membranes and (2) observations concerning the binding of diadenosine polyphosphates to purinoceptors in other tissues.

INTRODUCTION

Extracellular adenine nucleotides influence a variety of biological phenomena. This is a consequence of their interaction with specific purinoceptors on appropriate target tissues [1–3]. Diadenosine polyphosphates (Ap_nA; where $n = 3$–6) have now been identified in a wide variety of mammalian and other cells [4]. These unusual nucleotides account for a significant proportion of the nucleotide content of a range of storage granules including dense granules of platelets [5], secretory granules of chromaffin cells [6,7] and neuronal synaptic granules [8,9]. Discharge of these granules results in the liberation of diadenosine polyphosphates into the external environment from where they may, like ATP and other mononucleotides, exert effects on a diverse range of cell types [10–12]. Compared with mononucleotides, the diadenosine polyphosphates are characterized *in vivo* by relatively long half-lives and may therefore be suited to a role as long-range signalling molecules, exerting effects on target cells relatively distant from the site of their release into the extracellular space [5].

Among the better characterized targets for extracellular diadenosine polyphosphates are cells of the vasculature. Diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) inhibits ADP-induced platelet aggregation [13] and, in this context, the usefulness of synthetic analogues of Ap_4A as anti-thrombotic agents has recently been investigated [14]. Ap_4A and its homologues, Ap_3A, Ap_5A and Ap_6A, have also been implicated in the modulation of vascular tone and, hence, the control of blood pressure [15,16]. Additionally, Ap_3A and Ap_4A can prime the respiratory burst and regulate apoptosis in neutrophils [17,18].

Recent work has indicated that the diadenosine polyphosphates can also promote alterations in liver cell metabolism. Both Ap_3A and Ap_4A cause a transient release of Ca^{2+} and stimulation of glucose output from the perfused liver [19]. Using isolated rat liver cells we have recently shown that both these effectors, and a series of related phosphorothioate analogues, promote a dose-dependent stimulation of glycogen phosphorylase activity [20]. These effects appear to be a consequence of the ability of the diadenosine polyphosphates to promote oscillations in liver cell cytosolic free [Ca^{2+}] which closely resemble those seen in liver cells exposed to ATP or ADP [21].

The nature of the receptor(s) mediating the cellular signalling effects of diadenosine polyphosphates is unclear. Ap_4A has been reported to recognize the same population of receptors as ATP in sensory neurons [22]. On the basis of rank order potency experiments, other groups have attempted the classification of the receptor(s) binding diadenosine polyphosphates into one or more of the known P_2 purinoceptor subtypes [23–25]. On the other hand the presence of a 'unique' Ap_4A receptor (P_{2d}) in murine tissues [26–28] has been suggested, while other reports describe both P_{2d} [29] and P_4 [30] receptors in rat synaptic vesicles which show distinct diadenosine polyphosphate-binding characteristics. The purpose of the present investigation was to establish the relationship between the receptors recognized by Ap_4A and the known populations of purinoceptors in liver cell membranes and therefore to establish the relationship between ATP and Ap_4A-mediated signalling processes in rat liver cells.

MATERIALS AND METHODS

Chemicals

Percoll, Cibacron Blue 3GA, Basilen Blue E-3G, adenosine 5′-[$\alpha\beta$-methylene]triphosphate (pp[CH_2]pA), adenosine 5′-[$\beta\gamma$-methylene]triphosphate (p[CH_2]ppA), adenosine, AMP, ADP, ATP, adenosine 5′-[α-thio]triphosphate (ATP[S]), Ap_4, Ap_2A, Ap_3A, Ap_4A, Ap_5A and Ap_6A were obtained from the Sigma Chemical Co. Ltd., Poole, Dorset, U.K. 2-Methylthio-ATP was

Abbreviations used: Ap_2A, diadenosine 5′,5‴-P^1,P^2-diphosphate; Ap_3A, diadenosine 5′,5‴-P^1,P^3-triphosphate; Ap_4A, diadenosine 5′,5‴-P^1,P^4-tetraphosphate; Ap_5A, diadenosine 5′,5‴-P^1,P^5-pentaphosphate; Ap_6A, diadenosine 5′,5‴-P^1,P^6-hexaphosphate; Ap_4, adenosine 5′-tetraphosphate; ATP[S], adenosine 5′-[α-thio]triphosphate; ADP[S], adenosine 5′-[β-thio]diphosphate; pp[CH_2]pA, adenosine 5′-[$\alpha\beta$-methylene]triphosphate; p[CH_2]ppA, adenosine 5′-[$\beta\gamma$-methylene]triphosphate; NECA, *N*-ethylcarboxyamidoadenosine; R-PIA, (*R*)-phenylisopropyladenosine.

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from I.C.N. Pharmaceuticals Ltd., Thame, Oxfordshire, U.K. Collagenase, 5′-*N*-ethylcarboxamidoadenosine (NECA) and (*R*)-phenylisopropyladenosine (R-PIA) were from the Boehringer Corp. (London), Lewes, Sussex, U.K. [2-^{3}H]ATP (30 Ci/mmol) was obtained from Amersham International, Amersham, Bucks., U.K. and was used for the synthesis of [2-^{3}H]Ap_4A (30 Ci/mmol) as described previously [31]. Optiphase Safe scintillation fluid was from Pharmacia-LKB Biotechnology, Milton Keynes, U.K. All other chemicals were of the purest grade available from standard suppliers.

Animals

Male Wistar rats (University of Liverpool breeding colony) weighing 180–220 g were used throughout. Animals were fed *ad libitum* [Labsure Animal Diet (CRM); C. Hill Group, Poole, Dorset, U.K.].

Preparation and incubation of isolated liver cells

Liver cells were prepared as described previously [32]. Cells were incubated at 37 °C in Krebs-Henseleit medium [33]. In all cases, the incubation medium was supplemented with 2.5 mM $CaCl_2$ and 2 % (w/v) BSA. Incubation volumes were 0.4 ml throughout. In all experiments, metabolic integrity was assessed by measurement of cellular ATP content [34].

Preparation of liver cell plasma membranes

Liver cell plasma membranes were prepared essentially as described by Nakamura et al. [35]. Freshly isolated liver cells were homogenized, using a Ystral disperser, in ice-cold buffer containing 0.25 M sucrose, 2 mM EGTA and 10 mM Tris/HCl, pH 7.5. The homogenate was centrifuged at 1000 ***g*** for 30 s at 4 °C. The resulting supernatant was retained and the pellet was resuspended and recentrifuged as above. Supernatants were combined and centrifuged at 5000 ***g*** for 10 min at 4 °C. The resulting pellet was resuspended in buffer (see above), rehomogenized, and subsequently resuspended in a Percoll-containing buffer (7 vol. Percoll:1 vol. 2 M sucrose, 8 mM EGTA and 80 mM Tris/HCl, pH 7.5: 32 vol. 0.25 M sucrose, 2 mM EGTA and 10 mM Tris/HCl, pH 7.5). The resulting suspension was centrifuged at 10000 ***g*** for 20 min at 4 °C and the resulting plasma membrane fraction was collected. Membranes were subsequently washed repeatedly by centrifugation at 1000 ***g*** for 10 min at 4 °C using 10 mM Tris/HCl, pH 7.5, containing 0.15 M NaCl. Washed membranes were stored in 50 mM Hepes, pH 7.5, at −70 °C.

Binding assays

Ap_4A binding was assayed essentially as described by Hilderman et al. [26]. Briefly, each binding assay (final volume 620 μl) contained 67 mM Tris/HCl, pH 7.7, 100 μM $MgCl_2$ and 0.033 μM [2-^{3}H]Ap_4A (specific radioactivity 9000–10000 c.p.m./pmol). Binding was initiated by the addition of membrane fractions (10 μg) and incubations, in triplicate, were continued for up to 20 min at 25 °C. Incubations were terminated by collection of membranes on Whatman GF/C glass-microfibre filter discs, which were subsequently washed with 67 mM Tris/HCl, pH 7.7, containing 100 μM $MgCl_2$. The discs were counted for radioactivity in the presence of 10 ml of Optiphase Safe scintillation fluid. Blanks contained all components except membrane protein.

ATP binding was assayed as described above using 0.033 μM [2-^{3}H]ATP (specific radioactivity 9000–10000 c.p.m./pmol). In this case incubations were, unless stated otherwise, continued for 2 min at 25 °C before termination as described above.

In all assays non-specific binding was estimated from the extent of binding of the radiolabelled ligand in the presence of 1 mM ATP. This was routinely $< 2\,\%$ of total binding.

HPLC analysis

In certain experiments, the extent of breakdown of [2-^{3}H]ATP or [2-^{3}H]Ap_4A, in portions of incubation medium, was analysed by HPLC. Following incubation in the presence of either of these compounds, a 200 μl portion of medium was mixed with an equal volume of 0.4 M trichloroacetic acid and 400 μl of 0.6 M trioctylamine in Arcton (1,1,2-trichlorotrifluoroethane). After vigorous shaking, the mixture was centrifuged at 12000 ***g*** for 2 min at room temperature. Portions of the resulting upper aqueous layers were subjected to ultrafiltration (0.2 μm-pore-size Millipore filter) before HPLC analysis. Nucleotide breakdown products were separated by Bio-Gel MA7Q ion-exchange chromatography. Samples (100 μl), prepared as described above, were injected on to the column and then eluted using a linear gradient (21 mM–560 mM) of NH_4HCO_3, pH 9.6. Fractions (400 μl) were collected and 100 μl portions were counted for radioactivity in 3 ml of scintillation fluid.

Other assays

Protein concentrations were determined using the method of Bradford [36]. Binding data were analysed using the *P.Fit* curve-fitting program (Biosoft, Cambridge, U.K.).

RESULTS AND DISCUSSION

Stability of Ap_4A and ATP during binding assays

The ability to perform binding assays without significant breakdown of the ligand is particularly important in the context of the present experiments, since many of the potential breakdown products of Ap_4A (e.g. ATP, ADP, AMP and adenosine) are significant agonists of a range of different purinoceptor subtypes which have already been identified on liver cell plasma membranes. Figure 1 shows HPLC analyses of the extent of [2-^{3}H]Ap_4A and [2-^{3}H]ATP breakdown, in the presence of liver cell plasma membrane preparations, incubated under the conditions used for the analysis of nucleotide binding. These results clearly indicate that over the maximum time course used in the binding assays (20 min) there was no significant breakdown of the [2-^{3}H]Ap_4A with $> 95\,\%$ of the radiolabel co-migrating with the authentic compound. In contrast, in incubations where [2-^{3}H]ATP was the ligand, significant quantities of ADP accumulated over the incubation period with only $\sim 65\,\%$ of the radiolabel co-migrating with the ATP standard after 20 min. These results confirm that, under the conditions of our binding assays, breakdown of [2-^{3}H]Ap_4A was not a significant process and therefore binding can be attributed to the intact ligand. These findings are consistent with our previous observations concerning the stability of these nucleotides in incubations with intact liver cells [20] and with the findings of other workers who have shown that the diadenosine polyphosphates are characterized *in vivo* by relatively long half-lives in comparison with ATP [5].

Binding characteristics of Ap_4A

The binding of [2-^{3}H]Ap_4A to isolated liver cell plasma membranes was linear with membrane protein concentration (between

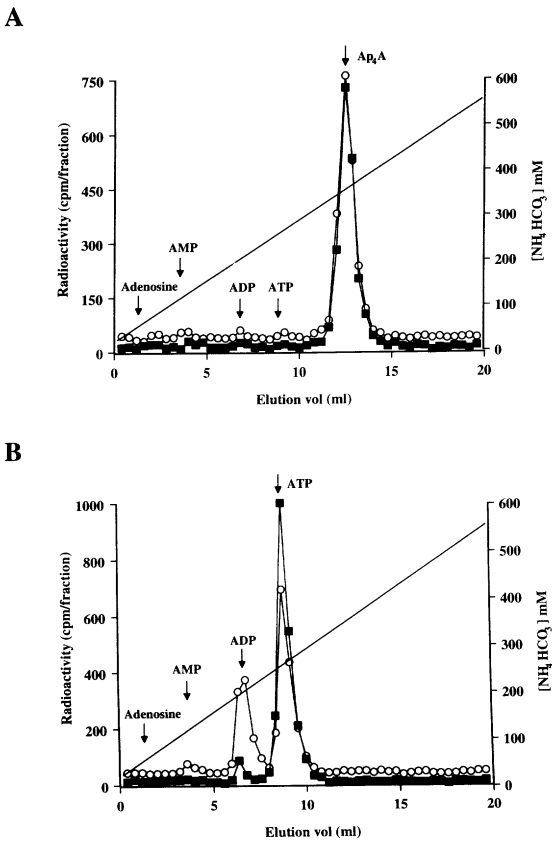

Figure 1 HPLC analysis of [2-^{3}H]Ap_4A and [2-^{3}H]ATP incubated in the presence of isolated liver cell plasma membranes

(**A**) Liver cell plasma membranes were incubated in the presence of 0.033 μM [2-^{3}H]Ap_4A (final specific radioactivity 9000–10000 c.p.m./pmol) for the indicated times. (**B**) Plasma membranes were incubated in the presence of 0.033 μM [2-^{3}H]ATP (final specific radioactivity 9000–10000 c.p.m./pmol) for the indicated times. Samples, prepared as described in the Materials and methods section, were subjected to HPLC analysis. The elution positions of authentic compounds are indicated by arrows and the linear NH_4HCO_3 gradient is also shown. Results are from a representative experiment. Key to symbols: ■, after 0 min incubation; ○, after 20 min incubation.

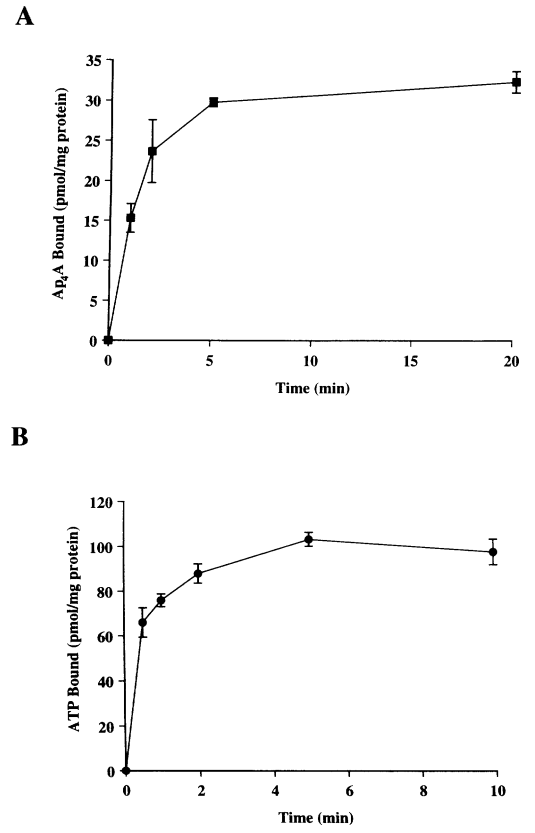

Figure 2 The time course of nucleotide binding to isolated liver cell plasma membrane preparations

The time course of binding of (**A**) 0.033 μM [2-^{3}H]Ap_4A and (**B**) 0.033 μM [2-^{3}H]ATP, to isolated liver cell plasma membranes (10 μg), was determined using the binding assay described in the Materials and methods section. Results are means ± S.D. for a representative experiment (n = three replicates) in each case.

5 and 30 μg of protein; results not shown). The time course of binding of [2-^{3}H]Ap_4A to isolated liver cell plasma membranes is illustrated in Figure 2. These results indicated that maximal binding (47 ± 7.4 pmol/mg of protein; mean ± S.E.M. for 11 independent membrane preparations) was attained after approx. 2–5 min. This was similar to the behaviour of [2-^{3}H]ATP; the binding of which showed maximal binding (86 ± 14 pmol/mg of protein; mean ± S.E.M. for four independent membrane preparations) after approx. 2 min (Figure 2). The maximal specific binding capacities for both Ap_4A and ATP were similar to those observed by other workers [26,37]. Analysis of the binding of [2-^{3}H]Ap_4A to intact liver cells indicated a broadly similar time course (results not shown). Although other workers have suggested that the binding of Ap_4A to crude membrane extracts is relatively slow, only attaining a maximal, saturable level after 1 h [26], the present observations are entirely consistent with the rapid effects (< 2 min) of Ap_4A and ATP on liver cell calcium homoeostasis and glycogen phosphorylase activation that we have previously observed [20,21].

Binding of [2-^{3}H]Ap_4A to isolated liver cell plasma membranes was reversible throughout the time course studied. The bound [2-^{3}H]Ap_4A was effectively displaced by the addition of an excess of unlabelled ligand (see later), indicating that there was no significant component of irreversibly bound ligand, a phenomenon which has been reported by other workers using prolonged (> 1 h) incubations with crude membrane preparations [26]. In an attempt to determine binding parameters for Ap_4A, experiments were carried out in which variable amounts of [2-^{3}H]Ap_4A, at constant specific radioactivity, were incubated in the presence of a fixed (10 μg) concentration of membrane protein (Figure 3). Application of the *P.Fit* curve-fitting program indicated that it was possible to interpret Ap_4A binding on the basis of a single specific binding site (~ 970 pmol of receptor/mg of membrane protein) with an apparent K_d of 1.76 ± 0.54 μM, a value similar to that reported for Ap_4A binding to murine membrane extracts [26]. An analogous investigation of [2-^{3}H]ATP binding to the same membrane preparations (results not shown) also suggested the occurrence of a single specific binding component (~ 1650 pmol of receptor/mg of membrane protein) with an apparent K_d of 1.39 ± 0.49 μM, a value indistinguishable from that obtained for Ap_4A binding (see above) and similar to that reported for both ATP[S] and adenosine 5′-[β-thio]diphosphate (ADP[S]) binding to liver cell membrane preparations [37–39].

The apparent K_d values obtained for both Ap_4A and ATP binding were similar to the concentrations of ligand required for

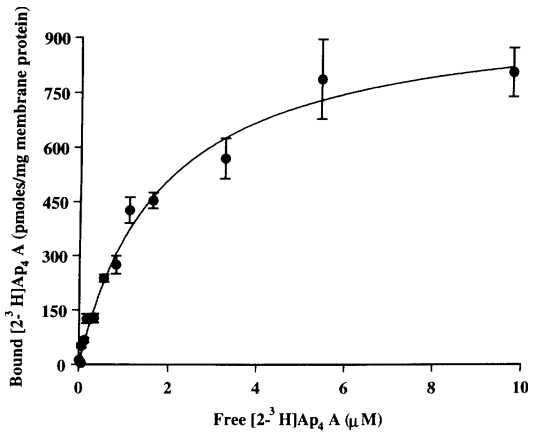

Figure 3 Analysis of [2-^{3}H]Ap_4A binding to isolated liver cell plasma membrane preparations

The binding of different concentrations of [2-^{3}H]Ap_4A, to isolated liver cell plasma membranes (10 μg), was determined using the binding assay described in the Materials and methods section. Curve fitting was carried out with the *P.Fit* computer program using a one-site, plus non-specific binding, model. Results are means ± S.E.M. for at least three independent plasma membrane preparations in each case.

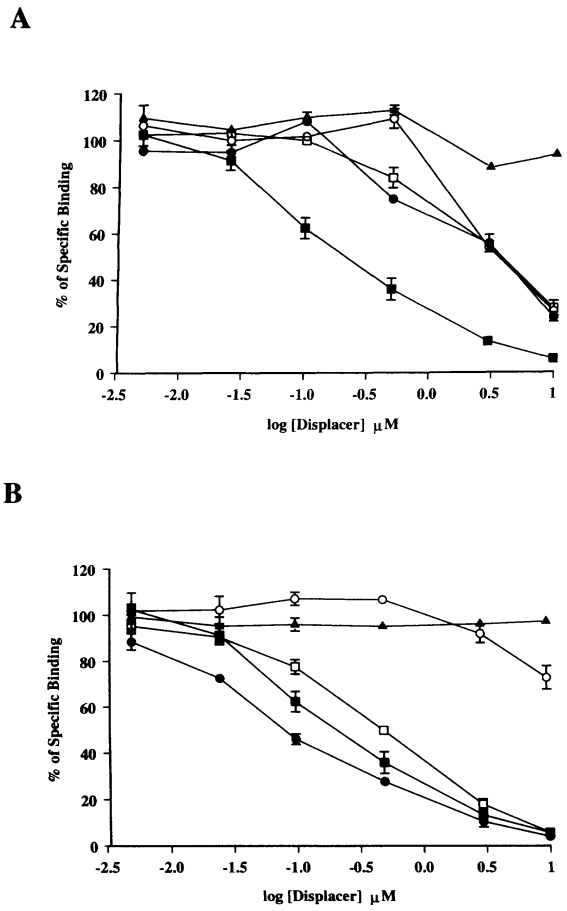

Figure 4 Displacement of bound [2-^{3}H]Ap_4A, from isolated liver cell plasma membrane preparations, by other nucleotides

The displacement of bound [2-^{3}H]Ap_4A from isolated liver cell plasma membrane preparations by (**A**) other diadenosine polyphosphates and (**B**) mononucleotides was determined using the binding assay described in the Materials and methods section. For (**A**): □, Ap_6A; ●, Ap_5A; ■, unlabelled-Ap_4A; ○, Ap_3A; and ▲, Ap_2A. For (**B**): ■, unlabelled-Ap_4A; ●, ATP; □, ADP; ▲, AMP; and ○, adenosine. Results are means ± S.E.M for at least three independent plasma membrane preparations in each case. Where error bars are not shown they are within the symbol.

half-maximal stimulation of glycogen phosphorylase activation in isolated liver cells [20], suggesting the absence of 'spare' receptors. The present binding experiments did not resolve the specific binding of either Ap_4A or ATP into more than one component (computer analysis of binding data did not support a two-site model for either ligand; results not shown). This is perhaps surprising in view of the evidence for the presence of distinct subclasses of purinoceptors in liver cell plasma membranes (see later); however, other workers have also failed to identify multiple, specific binding components using analogous binding studies [26,37,39]. In one case [38], the occurrence of two distinct, specific binding components for ADP[S] was reported; however, binding to the high-affinity component ($K_d \sim 7$ nM) was not correlated with any known biological effects. Taken together these observations suggest that, if distinct purinoceptor subtypes are responsible for Ap_4A and ATP binding in liver cell plasma membranes, the current binding studies were unable to resolve the binding parameters of these different receptor subtypes.

Specificity of Ap_4A binding

The ability of a wide range of naturally occurring and synthetic nucleotides to interfere with the binding of [2-^{3}H]Ap_4A to isolated liver cell plasma membranes was investigated. Figure 4 shows the effect of other diadenosine polyphosphates on Ap_4A binding to isolated liver cell membranes. These results indicated that both lower (Ap_2A, Ap_3A) and higher (Ap_5A, Ap_6A) homologues competed less effectively than increasing concentrations of unlabelled Ap_4A in the displacement of [2-^{3}H]Ap_4A from binding sites. These results were similar to those reported by other workers concerning binding of Ap_4A to murine brain membrane extracts [26]. Additionally, the relative ability of these compounds to displace Ap_4A was similar to their ability to stimulate activation of glycogen phosphorylase in isolated liver cells [20].

The ability of adenine mononucleotides to displace bound [2-^{3}H]Ap_4A was also assessed (Figure 4). These results indicated that ATP, adenosine 5′-tetraphosphate (Ap_4) (results not shown) and, to a lesser extent, ADP were effective displacers of bound [2-^{3}H]Ap_4A. In contrast, AMP and adenosine were completely ineffective. Therefore there appears to be a distinction between P_2-purinoceptor-preferring ligands (ATP and ADP) and P_1-purinoceptor-preferring ligands (AMP and adenosine), with respect to their ability to displace [2-^{3}H]Ap_4A from binding sites on isolated liver cell plasma membranes. The inability of P_1-purinoceptor-binding ligands to interfere with Ap_4A binding was confirmed in displacement experiments involving the adenosine analogues R-PIA (an A_1 agonist) and NECA (an A_2 agonist) (results not shown). This distinction between the displacing activity of P_1 and P_2 agonists differed from the observations of other workers, who reported that ATP and ADP showed only a very weak ability to displace Ap_4A bound to murine tissue extracts [26]. However, it should be pointed out that over the time course of the latter studies (> 1 h), the potential for breakdown of the competing ligand (possibly to AMP and adenosine) is considerable.

In view of the ability of P_2-purinoceptor agonists to displace [2-^{3}H]Ap_4A from isolated liver cell plasma membrane binding sites, we investigated the ability of a range of P_2 agonists and

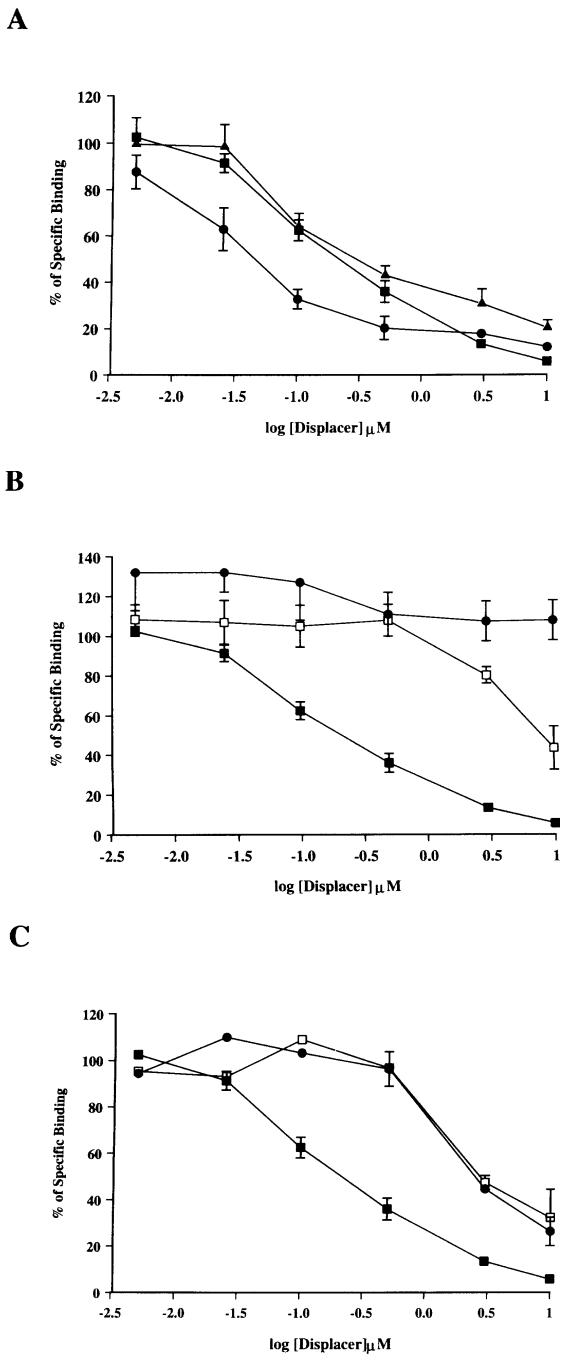

Figure 5 Displacement of bound [2-^{3}H]Ap_4A, from isolated liver cell plasma membrane preparations, by P_2-purinoceptor effectors

(**A**) Displacement of bound [2-^{3}H]Ap_4A, from isolated liver cell plasma membrane preparations, by 2-methylthio-ATP and UTP was determined using the binding assay described in the Materials and methods section. Key to symbols: ■, unlabelled Ap_4A; ●, 2-methylthio-ATP; ▲, UTP. (**B**) Displacement of bound [2-^{3}H]Ap_4A, from isolated liver cell plasma membrane preparations, by pp[CH_2]pA and p[CH_2]ppA was determined. (■), Unlabelled Ap_4A; (□), pp[CH_2]pA and (●) p[CH_2]ppA. (**C**) Displacement of bound [2-^{3}H]Ap_4A, from isolated liver cell plasma membrane preparations, by Cibacron Blue 3GA and Basilen Blue E-3G was determined. (■), Unlabelled Ap_4A; (□) Cibacron Blue 3GA and (●) Basilen Blue E-3G. Results are means ± S.E.M for at least three independent plasma membrane preparations in each case. Where error bars are not shown they are within the symbol.

antagonists to displace bound [2-^{3}H]Ap_4A (Figure 5). 2-Methylthio-ATP, which has previously been used as a specific agonist of the P_{2y}-purinoceptor subclass [40], was at least as effective as ATP as a displacer of [2-^{3}H]Ap_4A. In contrast, pp[CH_2]pA and p[CH_2]ppA, which show higher affinities for P_{2x} rather than P_{2y}-purinoceptor subtypes [41], were relatively ineffective at displacing [2-^{3}H]Ap_4A. Taken together these results indicated, for the displacement of [2-^{3}H]Ap_4A, a rank order potency of 2-methylthio-ATP > ATP ⩾ pp[CH_2]pA > p[CH_2]ppA. This is consistent with the order of displacement expected for a P_{2y}-like receptor, but is quite distinct from that reported for P_{2d}-like receptors in certain neural tissues [29]. The dye Reactive Blue 2 has previously been widely used as a P_{2y}-purinoceptor agonist [42]. We were unable to obtain this dyestuff as originally described; however, its major components, Cibacron Blue 3GA and Basilen Blue E-3G, were able to antagonize, albeit at rather high concentrations, [2-^{3}H]Ap_4A binding to liver cell membrane preparations (Figure 5).

The ability of UTP to interfere with Ap_4A binding was also investigated. As a consequence of the rapid breakdown of this compound under the conditions of the binding assay, these displacement studies were carried out over a maximum time course of 2 min. Figure 5 indicates that under these conditions UTP was almost as effective as unlabelled Ap_4A in the displacement of bound [2-^{3}H]Ap_4A. Previous studies by other workers have indicated that extracellular ATP and UTP have indistinguishable effects on isolated liver cell metabolism [43]. Where differences have been noted, in experiments involving the isolated perfused liver, these appear to be a consequence of differences in the ability of ATP and UTP to induce release of thromboxane from non-parenchymal cells [44]. UTP-specific purinoceptor subtypes (P_{2u}) have been characterized [41] and a recent report [45] has suggested that a cloned human P_{2u} receptor, although showing little affinity for 2-methylthio-ATP, readily binds Ap_4A. The ability of both 2-methylthio-ATP (see above) and UTP to compete with Ap_4A in the present experiments suggests that either UTP recognizes the same P_{2y}-like purinoceptor subtype recognized by Ap_4A or, alternatively, Ap_4A additionally recognizes P_{2u} receptors present in liver plasma membrane.

In addition to displacement studies with [2-^{3}H]Ap_4A, we also carried out a limited number of analogous experiments with [2-^{3}H]ATP over a 2 min time course (see above). These experiments indicated that the displacing abilities of 2-methylthio-ATP, ATP, Ap_4A, UTP and ADP were similar to that observed in our displacement experiments with [2-^{3}H]Ap_4A (results not shown). We also carried out a limited number of experiments relating to the displacement of bound [2-^{3}H]Ap_4A from either intact isolated liver cells (see Figure 6) or, alternatively, from membrane fractions prepared from whole liver (results not shown). In each case, the relative ability of ligands to displace bound [2-^{3}H]Ap_4A was identical to that observed using the isolated liver cell plasma membrane preparations described above. In particular, Figure 6 shows that Ap_4A, ATP and ATP[S] were similarly effective displacers of bound [2-^{3}H]Ap_4A from intact, isolated liver cells.

Conclusions

The experimental approaches described in this paper were designed to provide information about the binding of Ap_4A to rat liver cell plasma membranes. In particular, the relationship between the binding site(s) recognized by this ligand and the known purinoceptor subtypes was of interest. The present data are consistent with the existence of specific binding sites for Ap_4A on liver cell membranes and presumably it is the occupation of these sites that leads to the manifestation of the signalling properties of Ap_4A in the intact liver cell. Although there are considerable similarites between the characteristics of the binding

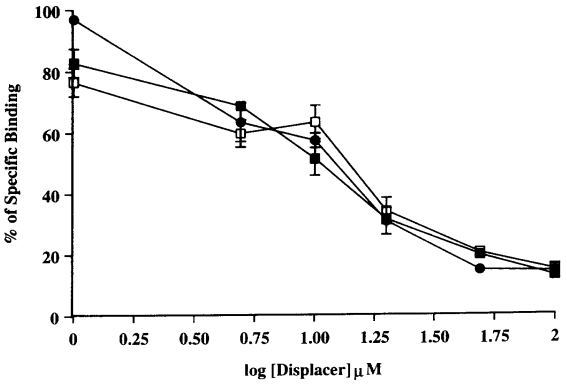

Figure 6 Displacement of bound [2-^{3}H]Ap_4A, from intact isolated liver cells by ATP and ATP[S]

Isolated liver cells were incubated in the presence of 0.033 μM [2-^{3}H]Ap_4A (final specific radioactivity 9000–10000 c.p.m./pmol) for 20 min. Displacement of the bound [2-^{3}H]Ap_4A by unlabelled nucleotides was determined using the binding assay described in the Materials and methods section. (■), Unlabelled Ap_4A; (●) ATP and (□) ATP[S]. Results are means ± S.E.M for at least three independent plasma membrane preparations in each case. Where error bars are not shown they are within the symbol.

processes that we have observed, and the nature of the binding of Ap_4A to other tissues that has been reported by other workers [26–28], there are also important differences. The present results clearly indicate that, for rat liver cells, there is a close relationship between the binding sites recognized by Ap_4A and those recognized by ATP. There is no evidence for the presence, in rat liver cell plasma membranes, of a subpopulation of binding sites unique for Ap_4A. This contrasts with the reported situation in murine tissues [26] and rat brain [30] where purinoceptor subtypes (P_{2d} and P_4 respectively) have been described which are apparently unique for the diadenosine polyphosphates, showing little affinity for ATP and other mononucleotide ligands. On the basis of the potency of analogues used in the original classification of purinoceptors [40,41] the present results are consistent with the Ap_4A binding sites of isolated liver cell plasma membranes being of the P_{2y}-subtype. This is analogous to the situation in bovine chromaffin cells [24,46] and Torpedo synaptosomes [8], which have been reported to bind diadenosine polyphosphates through P_{2y}-like purinoceptors.

Recently, it has become clear that there are probably distinct subpopulations of P_{2y}-subtype receptors in liver and other cell types [47]. In liver cells the coupling of distinct purinoceptors to diverse intracellular signalling pathways, leading to the inhibition of cyclic AMP accumulation or alternatively to the production of inositol trisphosphate [44,48], has been reported. Furthermore, on the basis of experiments involving analysis of the oscillations in cytosolic free [Ca^{2+}] induced by ATP and ADP, it now seems that there may be further heterogeneity among the population of P_{2y} receptors linked to inositol trisphosphate generation and Ca^{2+} mobilization [49,50]. Interestingly, the suggestion that Ap_4A binds a population of hepatic P_{2y}-purinoceptors which is also recognized by ATP is consistent with the results of our recent analysis of the oscillations in liver cell cytosolic free [Ca^{2+}] generated in response to Ap_4A [21]. The latter experiments indicated that whereas ATP was previously the only agonist to promote a variable oscillatory profile, this behaviour was also shown in response to Ap_4A. Furthermore, Ap_3A, which we found to show only weak displacer activity against Ap_4A (see above), did not display the variable [Ca^{2+}] oscillatory profile characteristic of ATP and Ap_4A; instead it showed an oscillatory profile very similar to that induced by ADP.

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