Characterization of the liver P_2-purinoceptor involved in the activation of glycogen phosphorylase

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Evidence has been presented for the existence in rat liver of P_2-purinoceptors which are involved in the control of glycogenolysis. Isolated rat hepatocytes and purified liver plasma membranes have been used to study the binding of the ATP analogue adenosine $5'$-[α-[^{35}S]thio]triphosphate (ATPα[^{35}S]) to these postulated P_2-purinoceptors. The nucleotide analogue behaves as a full agonist for the activation of glycogen phosphorylase in isolated hepatocytes, 0.3 μM being required for half-maximal activation. Specific binding of ATPα[^{35}S] to hepatocytes and plasma membranes occurs within 1 min and is essentially reversible. The analysis of the dose-dependency at equilibrium indicates the presence of binding sites with K_d of 0.23 μM with hepatocytes and K_d of 0.11 μM with plasma membranes. The relative affinities of 10 nucleotide analogues were deduced from competition experiments for ATPα[^{35}S] binding to hepatocytes, and these correlated highly with their biological activity (activation of glycogen phosphorylase in hepatocytes). For all the agonists, binding occurs in the same concentration range as the biological effect. These data clearly suggest that the detected binding sites correspond to the physiological P_2-purinoceptors involved in the regulation of liver glycogenolysis. The rank order of potency of some ATP analogues suggests that liver possesses the P_{2Y}-subclass of P_2-purinoceptors.

INTRODUCTION

Extracellular ATP and ADP influence many biological processes, such as platelet aggregation, cardiac function and muscular contraction (see Burnstock, 1981; Gordon, 1986). Mostly on the basis of an extensive pharmacological approach, the following consensus emerges. The nucleotides interact with specific receptors on the target cell surface and are ultimately degraded by ectonucleotidases. The purinoceptors (previously purinergic receptors) can be classified into P_1 and P_2 classes. P_1-receptors are more specific for adenosine, are blocked by methylxanthines and are positively linked to adenylate cyclase. P_2-receptors are more specific for ATP and ADP, are not blocked by methylxanthines and are not linked to adenylate cyclase. Burnstock & Kennedy (1985) have proposed to separate the P_2-purinoceptors into two subtypes, designated P_{2X} and P_{2Y}. The P_{2X} subtype shows a specific rank order of agonist potency (pp[CH$_2$]pA $\simeq$ p[CH$_2$]ppA > ATP = 2-methylthioATP) and is selectively desensitized by pp[CH$_2$]pA. The P_{2Y} subtype displays different rank order of potency (2-methylthioATP $\gg$ ATP > pp[CH$_2$]pA $\simeq$ p[CH$_2$]ppA), and is only weakly or not desensitized by pp[CH$_2$]pA.

Liver metabolism can also be regulated by submicromolar concentrations of ADP and ATP (see Keppens & De Wulf, 1985; Charest et al., 1985; Sistare et al., 1985). Both nucleotides induce the generation of myo-inositol 1,4,5-trisphosphate, the mobilization of Ca^{2+} (Charest et al., 1985) and the activation of glycogen phosphorylase (Keppens & De Wulf, 1985; Charest et al., 1985; Sistare et al., 1985), the rate-limiting enzyme of glycogenolysis. ATP and ADP (but not AMP or adenosine) share apparently a common mode of action with the cyclic AMP-independent Ca^{2+}-dependent glycogenolytic hormones angiotensin II and vasopressin and the α_1-adrenergic agonists (Keppens & De Wulf, 1985). The biological effects induced by ATP and ADP have been attributed to their interaction with P_2-purinoceptors (Keppens & De Wulf, 1985; Charest et al., 1985). Gordon (1986) tentatively proposed that liver P_2-purinoceptors belong to the P_{2Y} subclass.

We have now used ATPα[^{35}S] as a ligand, and we present data that strongly suggest that ATP and ADP interact with specific P_2-purinoceptors located in the plasma-membrane fraction of rat liver. The rank order of potency of some ATP analogues, established for biological activity and receptor affinity, indicates that liver P_2-receptors very likely belong to the P_{2Y} subclass.

EXPERIMENTAL

Materials

We used male Wistar-strain albino rats (200–250 g body wt.) that were fed ad libitum. Liver cells were isolated and incubated in a Krebs–Henseleit (1932) bicarbonate buffer equilibrated with O_2/CO_2 (19:1, v/v) as previously described (Vandenheede et al., 1976). Since no peptide agonists were studied, bacitracin was omitted from the incubation medium. For the preparation of liver plasma membranes, rats were killed by decapitation and the liver was homogenized with a Teflon/glass homogenizer (four strokes at 540 rev./min) in ice-cold 1 mM-NaHCO$_3$ (pH 7.4). The rest of the procedure was as described by Pilkis et al. (1974). The plasma membranes were kept in liquid N_2 until use.

ATPαS and ATPα[^{35}S] were purchased from Amer-

Abbreviations used: ATPαS, adenosine $5'$-[α-thio]triphosphate; ATPα[^{35}S], adenosine $5'$-[α-[^{35}S]thio]triphosphate; ATPγS, adenosine $5'$-[γ-thio]triphosphate; pp[CH$_2$]pA, adenosine $5'$-[α,β-methylene]triphosphate; p[CH$_2$]ppA, adenosine $5'$-[β,γ-methylene]triphosphate; p[NH]ppA, adenosine $5'$-[β,γ-imido]triphosphate; 2-methylthioATP, 2-methylthioadenosine $5'$-triphosphate.
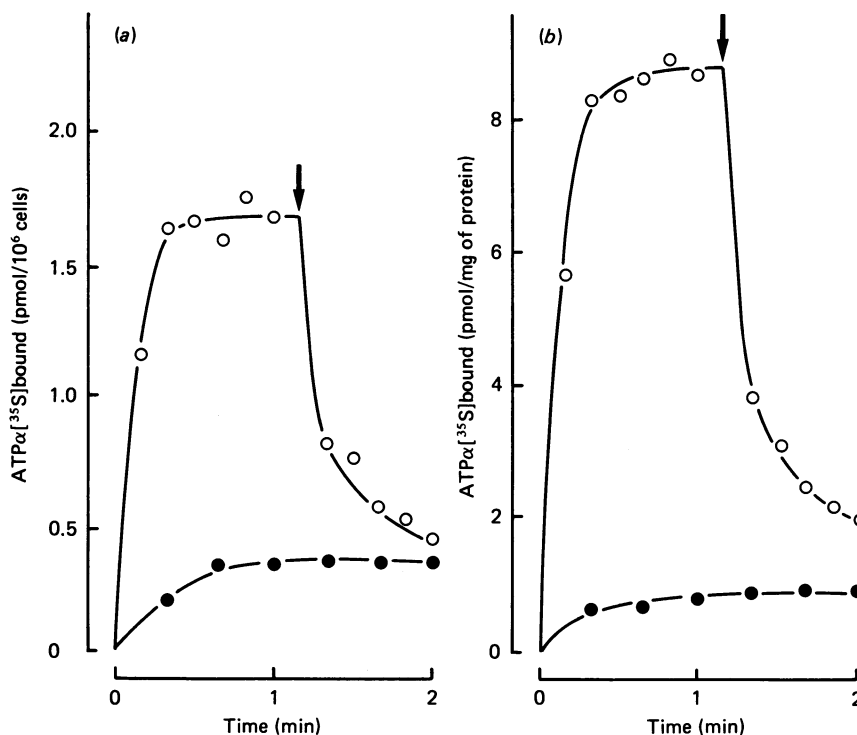
Fig. 1. Association–dissociation pattern for the binding of ATPα[^{35}S] to (a) hepatocytes or (b) liver plasma membranes

Hepatocytes (5×10^6/ml) or liver plasma membranes (0.6 mg of protein/ml) were incubated with 50 nM-ATPα[^{35}S], and 1 min later (arrow) an excess of ATP (1 mM) was added. Specific ($\bigcirc$) and non-specific ($\bullet$) binding are plotted as a function of time. This Figure shows results that are representative of five similar experiments.

sham International, Amersham, Bucks., U.K.; ATP was from Janssen, Beerse, Belgium; ATPγS and NADP$^+$ were from Serva, Heidelberg, Germany; ITP and GTP were from P-L Biochemicals, Milwaukee, WI, U.S.A; pp[CH$_2$]pA, p[NH]ppA, p[CH$_2$]ppA and ADP were from Sigma Chemical Co., St. Louis, MO, U.S.A. The sources of the other chemicals have been given previously (Keppens & De Wulf, 1984).

Analytical methods

Glycogen phosphorylase activity was determined as described by Vandenheede et al. (1976). Protein concentration was determined by the procedure of Bradford (1976) with bovine serum albumin as standard.

Biological response to ATPαS

ATPαS fully activates glycogen phosphorylase in isolated hepatocytes, even at slightly lower concentrations than ATP [see Fig. 3(a) in the Results section]; we have verified that, similarly to ATP, ATPαS achieves this activation in a cyclic AMP-independent Ca^{2+}-mediated way (results not shown). As reported previously (Keppens & De Wulf, 1985), the effect of ATP on the activation of glycogen phosphorylase is very transient, most probably owing to the degradation of the nucleotide by an ecto-ATPase (see Krell et al., 1983). The effect of ATPαS is less transient than that of ATP, suggestive of a slower degradation. This is in agreement with data indicating that the thio-ATP derivatives are more resistant to hydrolysis [see Stone (1985) for a discussion]. The stability of the nucleotide was analysed as previously described for angiotensin (Keppens et al.,

1982): incubation for 1 min with the hepatocytes resulted in a non-significant loss of glycogenolytic potency. Neither did we observe a decreased potency to displace ATPα[^{35}S] from its receptor, as analysed in Figs. 3(b) and 3(c).

Measurement of ATPα[^{35}S] binding to isolated hepatocytes

We used basically the same procedure as that described for our studies of receptors for vasopressin (Cantau et al., 1980) and angiotensin (Keppens et al., 1982). Cells (5×10^6) were preincubated for 20 min at 37 °C in 1 ml of Krebs–Henseleit bicarbonate medium with 10 mM-glucose. ATPα[^{35}S] was then added; at the times indicated, samples (25 μl) were withdrawn and collected in 4 ml of ice-cold 3 mM-Hepes buffer (pH 7.4) containing 0.15 M-NaCl, 2 mM-CaCl$_2$, 1.3 mM-MgCl$_2$ and 1 mM-ATP. These samples were immediately filtered at 0 °C on Whatman GFA filters pre-soaked in this buffer. The presence of ATP is mandatory to prevent spurious binding to the filters. These were washed with 3×7 ml of ice-cold 3 mM-Hepes buffer (pH 7.4) containing 0.15 M-NaCl. The whole procedure was performed within 10 s. It has been checked that this low temperature (0–4 °C) avoids dissociation of the bound ATPα[^{35}S]. Indeed, the same amount of bound ATPα[^{35}S] was detected when samples were treated as described or were kept for 30 s at 0 °C in the stopping buffer before filtration. The filters were dried in the open air and their radioactivity was counted by liquid-scintillation spectrometry. Non-specific binding was determined by adding a 200-fold excess of ATP over ATPα[^{35}S] to the incubation
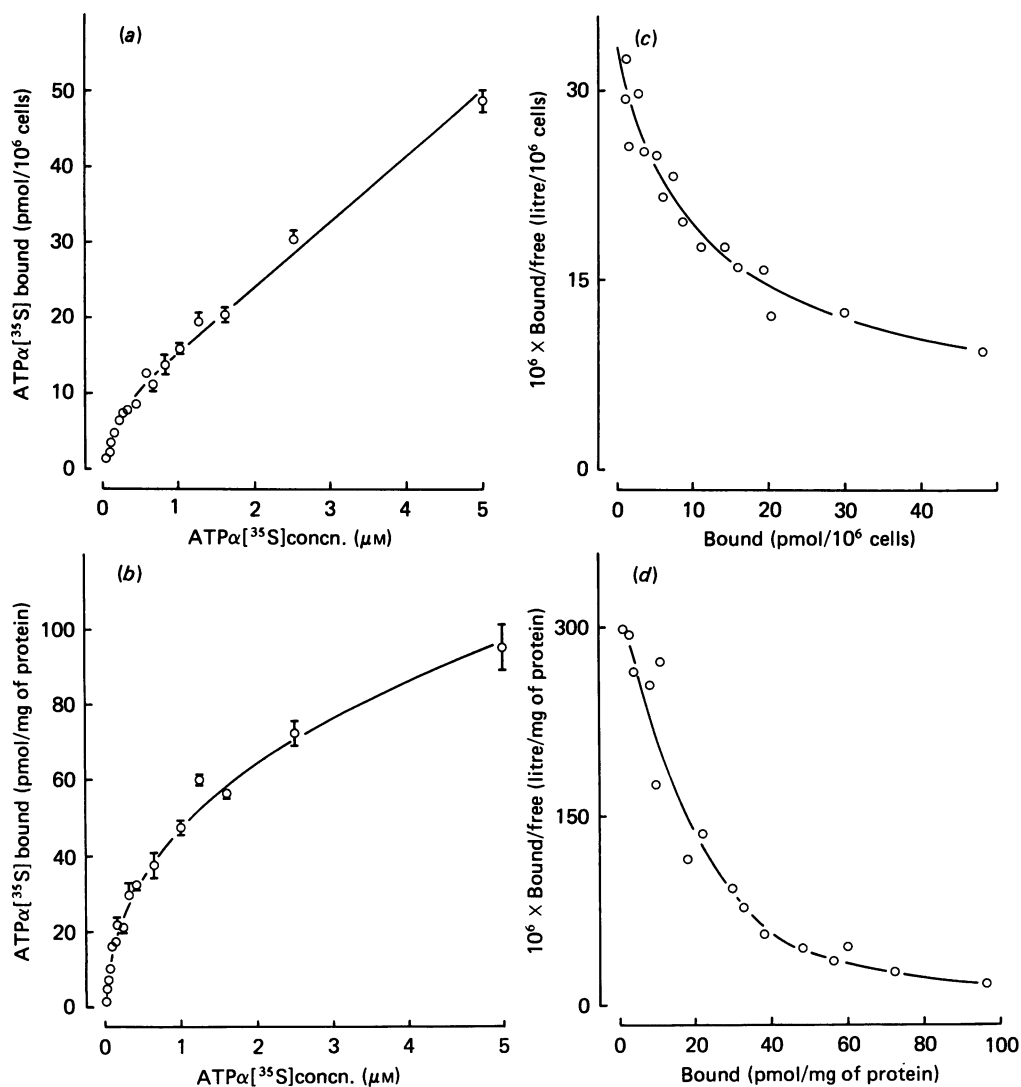
Fig. 2. Dose-dependency of specific ATPα[³⁵S] binding to isolated hepatocytes and purified liver membranes

(*a*) and (*b*), Values for specific binding after 1 min of incubation given as means ± S.E.M. (where scale permits) for three to four experiments. (*c*) and (*d*), Scatchard (1949) plots of the data of (*a*) and (*b*) respectively. As explained in the text, the data can be accounted for on the basis of the existence of a class of high-affinity binding sites and of a non-saturable component. K_d and B_{max} values are given in the text.

mixture. The binding of ATPα[³⁵S] to hepatocytes increased linearly with the cell concentration tested up to 10^7 cells/ml. The dissociation constants of the ATP analogues were deduced from competition experiments with 50 nM-ATPα[³⁵S] and the unlabelled analogues, as described previously for the vasopressin receptor (Cantau *et al.*, 1980).

Measurement of ATPα[³⁵S] binding to purified plasma membranes

Membranes (corresponding to 60 μg of protein) were incubated at 37 °C in 100 μl containing ATPα[³⁵S] in 24 mM-Tris/HCl buffer (pH 7.4)/1.5 mM-MgCl₂. At the times indicated, the binding was stopped by adding 4 ml of ice-cold 3 mM-Hepes buffer (pH 7.4) containing 0.15 M-NaCl, 2 mM-CaCl₂, 1.3 mM-MgCl₂ and 1 mM-ATP (as discussed above for cells). The subsequent procedure was as described above for cells.

RESULTS AND DISCUSSION

Fig. 1 illustrates the association–dissociation patterns of ATPα[³⁵S] binding to hepatocytes and plasma membranes. Specific binding was very fast and was essentially reversible on addition of an excess of ATP. Consequently, steady-state binding measurements were performed after an incubation period of 1 min. The dose-dependencies obtained for the binding of ATPα[³⁵S] to hepatocytes or membranes, as shown in Figs. 2(*a*) and 2(*b*), cannot adequately be described by a Michaelian-type relationship. Scatchard (1949) plots of the binding data are in both cases curvilinear (Figs. 2*c* and 2*d*). It has been computed that these curvilinear Scatchard plots can be separated into two independent components. The first represents saturable binding, characterized by a specific K_d and B_{max}. It is shown below that this component very likely corresponds to the physiological receptor (see below). With cells, the computed K_d and B_{max} values for

Table 1. Comparison of the pK_a and pK_d values of nucleotide analogues

Apparent affinity constants (K_a) for phosphorylase activation (see Fig. 3a) were computed as described by Bréant et al. (1981) and expressed in terms of pK_a ($-\log K_a$). All analogues used were full agonists. Dissociation constants for the binding were calculated from data obtained in competition experiments (see Figs. 3b and 3c) and expressed as pK_d. Values listed are means ± s.D. (n).

Nucleotide	pK_a	pK_d
1. ATPαS	6.62 ± 0.10 (5)	6.64 ± 0.21 (3)
2. ADP	6.46 ± 0.13 (5)	6.10 ± 0.07 (3)
3. ATPγS	6.20 ± 0.22 (3)	5.80 ± 0.12 (4)
4. ATP	6.30 ± 0.25 (6)	5.74 ± 0.24 (7)
5. p[CH₂]ppA	5.50 ± 0.08 (3)	4.95 ± 0.23 (4)
6. p[NH]ppA	5.64 ± 0.35 (3)	4.80 ± 0.02 (3)
7. ITP	5.00 ± 0.39 (4)	4.75 ± 0.03 (3)
8. GTP	4.92 ± 0.22 (4)	4.64 ± 0.17 (3)
9. pp[CH₂]pA	4.35 ± 0.15 (3)	3.73 ± 0.08 (4)
10. NADP⁺	3.40 ± 0.28 (3)	3.35 ± 0.17 (5)

the high-affinity binding site are 0.23 μM and 5 pmol/10⁶ cells. With membranes, the K_d and $B_{max.}$ values are 0.11 μM and 30 pmol/mg of protein. The number of receptors per cell is surprisingly high (about 3×10^6 sites per cell), but could be needed to compensate for the low affinity for ATP. In this context it is maybe meaningful

to point out that for vasopressin and for angiotensin the number of receptors/cell is about 10 times less than the number of receptors for ATP, but that their affinity for their receptors is about 10 times higher (Cantau et al., 1980; Keppens et al., 1982). The other component, obtained at higher concentrations of ATPα[³⁵S], can be represented by a 'non-saturable' binding component. Its physiological nature is not readily apparent, but, given the high concentrations, it is probably not of any physiological significance.

Next, experiments were designed to check the inherent assumption made that the high-affinity sites, revealed by ATPα[³⁵S] binding, represent the biological receptors that mediate the ATP-induced phosphorylase activation. With hepatocytes we have compared, for a series of ATP analogues, both their biological potencies and their affinities for the binding sites, as estimated by their abilities to prevent the binding of 50 nm-ATPα[³⁵S] to isolated hepatocytes; at this low concentration, ATPαS will predominantly bind to the high-affinity binding sites. For four analogues, the dose–response curves for liver glycogenolysis (a) and ligand displacement (b and c) are shown in Fig. 3. The respective glycogenolytic potencies and dissociation constants of all analogues studied are listed in Table 1. The close similarity between both values indicates that binding occurs in the same range as the biological effect, allowing the conclusion that no spare receptors are involved in the purinoceptor-mediated control of glycogenolysis.

Furthermore, the highly significant correlation between these two parameters ($r = 0.97$, $P < 0.001$), as
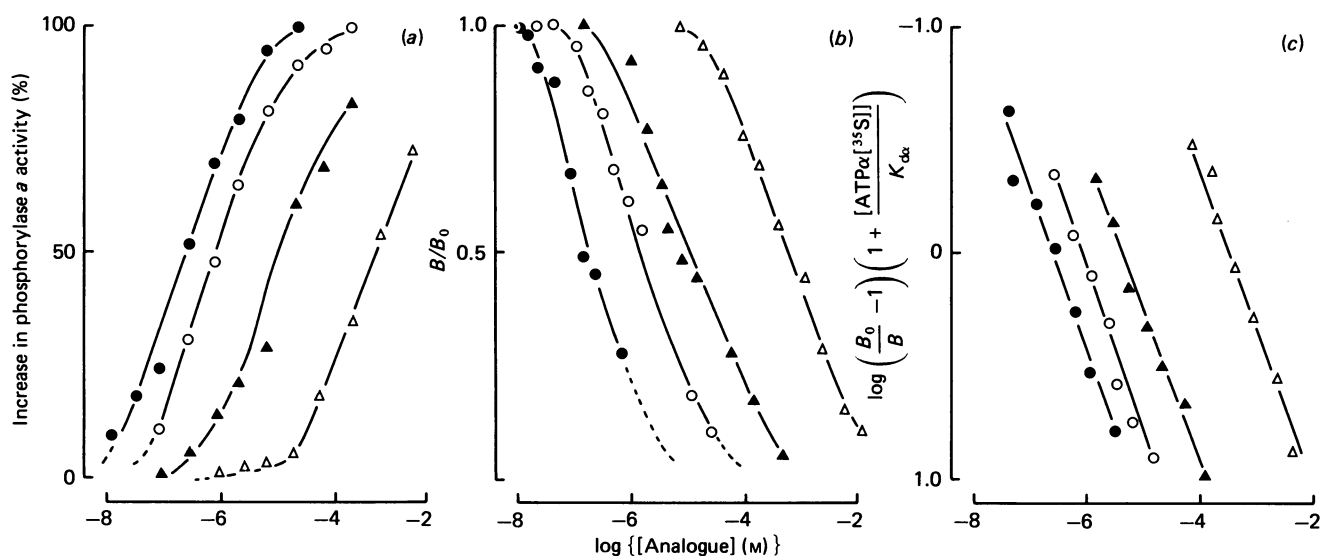
Fig. 3. Determination of glycogenolytic potencies and of dissociation constants for four nucleotides or analogues

(a) Liver cells were challenged with increasing concentrations of ATPαS (●), ATP (○), GTP (▲) or NADP⁺ (△). Phosphorylase a was assayed 20 s later. Basal values are about 20–25 munits/mg of protein: maximal values range from 90 to 100 munits/mg of protein. Results are expressed as % increase above basal values. (b) Specific binding of ATPα[³⁵S] (50 nm) was measured after 1 min in the presence of increasing amounts of unlabelled analogue. Values of specific binding measured in the presence of unlabelled analogue (B) were expressed as a fraction of the specific binding measured in the absence of competitor (B_0). (c) Dissociation constants for the unlabelled nucleotide, expressed as pK_d ($-\log K_d$) were deduced by fitting the experimental data obtained in (b) to the expected linear relationship

$$\log\left(\frac{B_0}{B} - 1\right)\left(1 + \frac{[\text{ATP}\alpha[^{35}\text{S}]]}{K_{d\alpha}}\right) = \log[\text{analogue}] - \log K_d$$

$K_{d\alpha}$, the K_d value for ATPα[³⁵S], was taken as 0.23 μM, characterizing the high-affinity binding site.
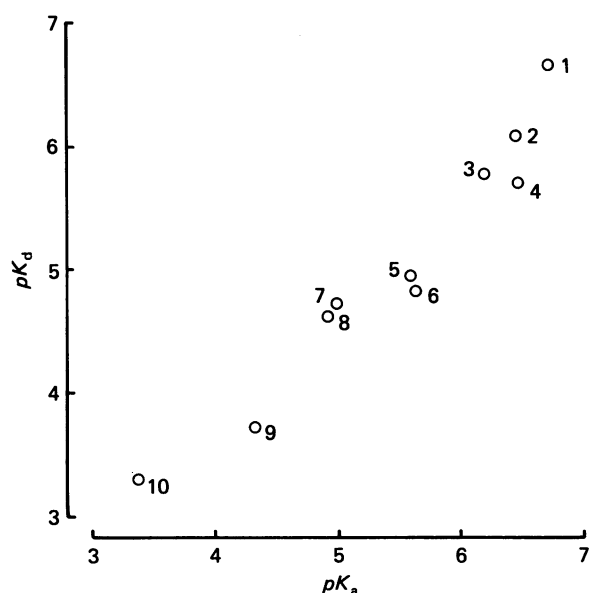
Fig. 4. Correlation between pK_a and pK_d values for the nucleotides and analogues

The numbers represent the analogues listed in Table 1 ($r = 0.93$; $P < 0.001$).

illustrated in Fig. 4, strongly suggests the involvement of these high-affinity binding sites in mediating the biological response of the liver to purinergic stimulation.

Methylisobutylxanthine, used at 0.1 mM, is unable to displace ATPα[^{35}S] from the high-affinity binding sites (results not shown) clearly indicating that, according to the criteria mentioned in the introduction, P$_2$-purinoceptors are involved.

The fact that the methylene derivatives of ATP (pp[CH$_2$]pA and p[CH$_2$]ppA) are less potent to cause activation of phosphorylase and have less affinity for the receptor than ATP (Table 1) is suggestive of the presence in liver of the P$_{2Y}$ subtype of the P$_2$-purinoceptor. A further argument for this proposal is given by our finding that pp[CH$_2$]pA is not capable of inducing a desensitization of the liver purinoceptors (results not shown) [see Burnstock & Kennedy (1985) for a review].

Conclusion

We have used ATPα[^{35}S] to characterize the liver P$_2$-purinoceptors. The analogue behaves as a full agonist in activating glycogen phosphorylase in isolated rat hepatocytes. Its binding to hepatocytes and purified liver plasma membranes is at apparent equilibrium after less than 1 min and is essentially reversible. The data indicate the presence of high-affinity binding sites. Their physiological significance is substantiated by the excellent correlation shown by ten nucleotides for their glyco-genolytic potencies and their dissociation constants with isolated cells. It is concluded that rat liver possesses specific P$_2$-purinoceptors, most probably of the P$_{2Y}$ subclass, involved in the control of glycogenolysis.

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