Br. J. Pharmacol. (1993), 108, 663–668

Characterization of the effects of adenosine 5′-[β-thio]-diphosphate in rat liver

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1 In rat liver cells micromolar concentrations of adenosine 5′-[β-thio]diphosphate (ADPβS), activate glycogen phosphorylase by an adenosine 3′:5′-cyclic monophosphate (cyclic AMP)- independent mechanism.

2 As with adenosine 5′-triphosphate (ATP), ADPβS also inhibits the rise in cyclic AMP after glucagon.

3 Cytosolic Ca^{2+} measured in single cells is rapidly increased with a pattern similar for ADPβS and for ATP.

4 At variance with ATP, ADPβS hardly increases inositol 1,4,5-trisphosphate (IP_3) levels.

5 Phorbol myristic acetate, which inhibits only slightly the glycogenolytic effect of ATP, almost completely abolishes this effect of ADPβS.

6 With adenosine 5′-[β-[^{35}S]thio]diphosphate (ADPβ[^{35}S]) as radioligand, we detected specific purinoceptors on rat liver plasma membranes. Binding consists of a major binding component with $K_D = 0.7\ \mu M$ and $B_{max} = 51$ pmol mg^{-1} of protein, probably mediating the activation of glycogen phosphorylase, and a minor high affinity, low capacity binding component with no obvious function.

7 It is concluded that the differences in biological effects between ATP and ADPβS may involve different receptors and/or different transduction mechanisms and that ADPβ[^{35}S] can be used to detect the specific binding sites for ADPβS.

Keywords: Purinoceptors; rat liver; glycogenolysis; adenosine 5′-[β-thio]diphosphate

Introduction

In isolated hepatocytes of the rat, extracellular adenosine 5′-triphosphate (ATP) and adenosine 5′-diphosphate (ADP) are able to activate glycogen phosphorylase, which is associated with a rapid increase in inositol 1,4,5-trisphosphate (IP_3) levels and intracellular Ca^{2+}(Keppens & De Wulf, 1985; Charest *et al.*, 1985; Sistare *et al.*, 1985; Okajima *et al.*, 1987). The purinoceptor agonists ATP and ADP, like the other Ca^{2+}-dependent agonists (vasopressin, angiotensin II and the α_1-adrenoceptor agonists) also inhibit the glucagon (Keppens & De Wulf, 1985) and forskolin (Okajima *et al.*, 1987)-induced rise in adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels.

The rank order of potency of several ATP-analogues in activating glycogen phosphorylase (Keppens & De Wulf, 1985) pointed to the presence of P_{2Y}-purinoceptors according to the basic nomenclature of Burnstock & Kennedy (1985) (ATP = ADP > α,β-methylene-ATP, β,γ-methylene-ATP). Using adenosine 5′-[α-[^{35}S]thio]triphosphate (ATPα[^{35}S]) as a radioligand, we have characterized the purinoceptors on rat liver parenchymal cells and plasma membranes (Keppens & De Wulf, 1986), in human liver plasma membranes (Keppens *et al.*, 1989) and in plasma membranes of rabbit and guinea-pig liver (Keppens *et al.*, 1990) and shown that the detected binding site corresponds to a P_{2Y}-type of purinoceptor, mediating the glycogenolytic effect. Extracellular UTP induces glycogenolysis in rat hepatocytes in a manner very similar if not identical to ATP, suggesting that both agonists bind to the same receptor (Keppens *et al.*, 1992). On the other hand, important differences in biological effects between 2-methylthio adenosine triphosphate (2MeSATP), known to be the most potent P_{2Y}-agonist in several tissues, and ATP have been demonstrated in rat liver cells (Keppens & De Wulf, 1991). Although 2MeSATP is indeed more potent in activating glycogen phosphorylase than is ATP, thereby fitting the P_{2Y}-classification, the following results, at variance with those obtained with ATP, suggest that 2MeSATP probably binds to another type of P_2-purinoceptor: (a) IP_3-levels are hardly increased; (b) no anti-glucagon effect on cyclic AMP levels could be detected; (c) addition of phorbol myristic acetate totally blocked the activation of phosphorylase. Therefore rat liver, like other cell types (O'Connor *et al.*, 1991; O'Connor, 1992), may contain a heterogeneous 'mixed' receptor population with the following rank order of potency for glycogenolysis: 2MeSATP > ATP = ADP = UTP.

In turkey erythrocytes, adenosine 5′-[β-thio]diphosphate (ADPβs) activates a guanine nucleotide-sensitive phospholipase C with a severalfold increase in inositol phosphates (Boyer *et al.*, 1989; Martin & Harden, 1989; Berrie *et al.*, 1989). Cooper *et al.* (1989) used adenosine 5′-[β-[^{35}S]thio]-diphosphate (ADPβ[^{35}S]) as radioligand to study the P_{2Y}-receptors present on turkey erythrocyte plasma membranes.

This study was undertaken to evaluate the use of ADPβS and ADPβ[^{35}S] as suitable agonist and radioligand for the rat liver P_{2Y}-purinoceptors.

Methods

We used male Wistar-strain albino rats (200–250 g body weight) that were fed *ad libitum*.

Liver cells were isolated and incubated in a Krebs-Henseleit bicarbonate buffer equilibrated with O_2/CO_2 (19:1, v/v) as previously described (Vandenheede *et al.*, 1976) but without bacitracin. Liver plasma membranes were prepared according to the method of Pilkis *et al.* (1974) with slight modifications (Keppens & De Wulf, 1985). The plasma membranes were kept at −80°C until use.

Glycogen phosphorylase activity was determined as described by Vandeheede *et al.* (1976).

Cyclic AMP and IP_3 were measured with a competitive protein-binding technique, the former (Gilman, 1970) by

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using an assay kit from the Radiochemical Centre (Amersham, Bucks, U.K.) and the latter as described by Bredt *et al.* (1989) with slight modifications (Keppens *et al.*, 1992).

Intracellular Ca^{2+} was estimated by Fura-2 fluorescence. Cells were loaded with the acetoxymethylester of Fura-2 by incubation of hepatocytes, 0.5 to 1×10^6 cell ml^{-1}, in a solution containing (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 10, glutamate 4.5, pyruvate 5, HEPES 20 and $CaCl_2$ 2 pH 7.4 and supplemented with 1% bovine serum albumen (BSA), 0.05% pluronic acid, 10 μM Fura-2-AM and 0.5% dimethylsulphoxide at 30°C for about 20 to 30 min. The cells were then extensively washed and transferred into a Petri dish with a thin glass bottom, lightly coated with polylysine. The dish was mounted on a microscope (inverted Nikon Diaphot), equipped with a dual excitation microspectrofluorimetric system (Newcastle Photometric System). Fura-2 was alternately excited at 340 and 380 nm and emission fluorescence was collected at 510 nm. Measurements were performed at room temperature. $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence according to the equation $[Ca^{2+}] = K_D{*}B{*}(R-R_{min})/R_{max}\text{-}R)$ (Grynkiewicz *et al.*, 1985), with $K_D = 224$ nM, B the ratio of the fluorescence signals obtained by illuminating the cells at 380 nM taken at 0 (EGTA) or saturating Ca^{2+}-concentrations (ionomycin, 4-bromo A23187). R represents the ratio at any time whereas R_{max} and R_{min} are the ratios obtained at saturating, zero calcium respectively. The autofluorescence never exceeded 10% of the fluorescence of a Fura-loaded cell and was subtracted from the total fluorescence.

ADPβ[^{35}S] binding to purified liver plasma membranes was carried out according to the method described for ATPα[^{35}S] (Keppens & De Wulf, 1985). Briefly, ADPβ[^{35}S] was incubated at 37°C with liver plasma membranes (± 0.6 mg of protein ml^{-1}); bound and unbound ligand were separated by filtration on a Whatman GF/A filter. Non-specific binding was estimated in the presence of an excess of ADP and never exceeded 12% of the total binding. Analysis of the binding parameters was done with the computer programme 'Enzfitter' (Leatherbarrow, 1987) or with the fitting facilities of the graphic package Fig.P, used for drawing the figures.

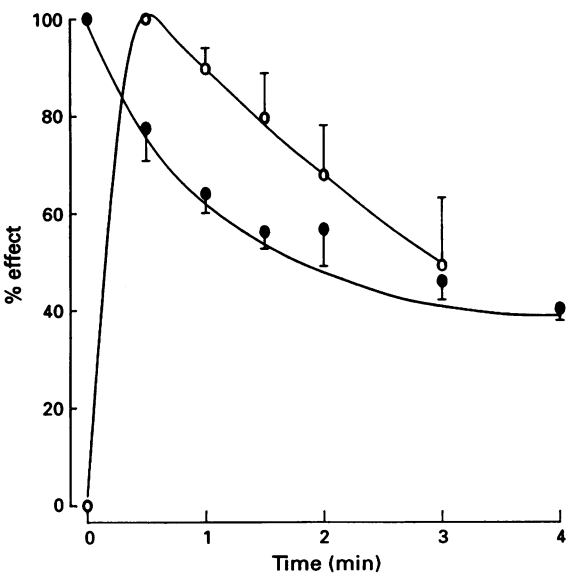

Figure 1 Time-dependent effect of adenosine 5'-[β-thio]diphosphate (ADPβS) on the activation of phosphorylase and of its inhibition of the cyclic AMP increase. Rat hepatocytes were preincubated at 37°C for 25 min with 10 mM glucose. For the activation of phosphorylase (○), 5 μM ADPβS was then added. Enzyme activity was determined at the indicated time points and is expressed as percentage of effect. Basal levels of phosphorylase *a* (10 ± 4 mu mg^{-1} of protein) were increased to 85 ± 5 mu mg^{-1} of protein (100%) after 30 s. For the inhibition of the cyclic AMP increase (●), cells were first challenged with 20 nM glucagon; 2 min later, 200 μM ADPβS was added and cyclic AMP levels were measured at the indicated time points. Basal values of cyclic AMP (4.1 ± 0.7 pmol mg^{-1} protein) were increased to 33 ± 3 pmol mg^{-1} protein (100%) 2 min after glucagon. Values are given as means ± s.e.mean ($n = 4$).

Materials

ADPβS (adenosine 5'-[β-thio]diphosphate) was from Boehringer, Mannheim (Germany), ADPβ[^{35}S] (adenosine 5'-[β-[^{35}S]thio]-diphosphate; specific activity 45.9 TBq $mmol^{-1}$, 1240 Ci $mmol^{-1}$) was purchased from New England Nuclear, Du Pont-NEN Research products, Brussels (Belgium). ATP, phorbol 12-myristic 13-acetate, ionomycin and collagenase type 1 were from Sigma Chemical Co., St. Louis, MO (U.S.A.). Fura 2 AM, pluronic acid and 4-bromo A23187 were from Molecular Probes Inc., Eugene, OR (U.S.A.). [^{3}H]-inositol-(1,4,5)-trisphosphate was from Amersham International, Amersham, Bucks. (U.K.). The sources of other chemicals have been given previously (Keppens *et al.*, 1989).

Results

We first compared some biological effects of ATP and ADPβS in rat hepatocytes in order to check whether the latter behaves as a P_2-purinceptor agonist.

Figure 1 shows the kinetics of ADPβS on the activation of glycogen phosphorylase and on the inhibition of the cyclic AMP rise after glucagon. Phosphorylase is already maximally activated after 30 s and declines afterwards. The anti-glucagon effect proceeds more slowly (maximum after about 2 min) but lasts longer. This is identical to what has been found for ATP (Keppens & De Wulf, 1985). The dose-dependent activation of phosphorylase (Figure 2a), estimated 30 s after the addition of increasing concentrations of ADPβS, reveals a K_a (half-maximal activation constant) value of ≈1 μM as was also obtained earlier with ATP (Figure 2a, dashed line, Keppens & De Wulf, 1985). The anti-glucagon effect of ADPβS is also similar to that of ATP (Figure 2b) with a K_i of about 20 μM, indicating that higher concentrations are needed to suppress the glucagon effect than to activate phosphorylase. Like ATP, ADPβS on its own does not influence cyclic AMP levels (not shown).

Since Ca^{2+} is proposed to be the main second messenger used by the P_2-purinoceptor agonists, we studied the rise in cytosolic Ca^{2+}. The experiments were performed on single hepatocytes to avoid the possibility that differences might be masked in whole cell suspensions. The cells were loaded with Fura 2 and superfused with 2 μM ADPβS or 2 μM ATP. A typical result is illustrated in Figure 3. The overall pattern of the Ca^{2+}-mobilization after the addition of both agonists is very similar. A rapid elevation in cytosolic Ca^{2+}can be detected, reaching a maximal value and then declining to a plateau, which persists as long as ATP or ADPβS is present. However, it should be noted that occasionally, some cells, clearly responsive to ATP, did not mobilize Ca^{2+} when superfused with ADPβS (not shown).

Next, we examined the generation of IP_3 by either agonist in the hepatocytes. Earlier studies (Charest *et al.*, 1985; Keppens *et al.*, 1992) revealed that higher concentrations of ATP are needed to increase IP_3 ($K_{IP} = 40$ μM) than to activate phosphorylase and that the IP_3-rise is very rapid (maximal after 5 s) and transient. However, when using ADPβS, it was found that only very high concentrations could augment IP_3-levels. Figure 4 shows the levels of IP_3, 5 s after 100 μM ATP or 100 μM ADPβS had been added. Clearly, ADPβS is far less able to generate the second messenger. It was checked that for ADPβS, a maximal response was also reached within 5 s. We checked that a 10 fold excess of ADPβS over ATP did not inhibit the rise of IP_3 normally observed after ATP (two independent experiments, data not shown).

To compare ATP and ADPβS further, we studied the effect of phorbol myristic acetate (PMA). PMA is known to in-

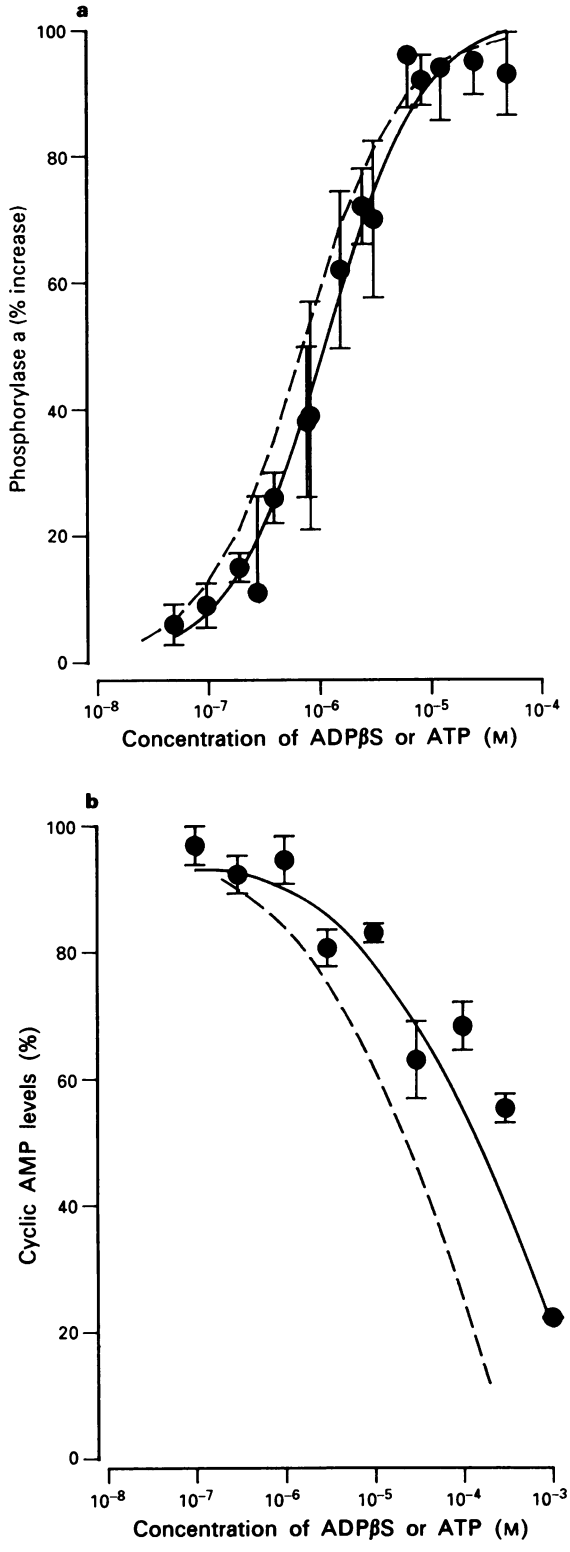

Figure 2 Dose-dependent effects of adenosine 5'-[β-thio]diphosphate (ADPβS) and ATP on the activation of glycogen phosphorylase and of the inhibition of the cyclic AMP increase. Rat hepatocytes were preincubated at 37°C for 25 min with 10 mM glucose. (a) They were then treated with increasing concentrations of ADPβS (●; solid line) for 30 s. The dashed line represents the theoretical curve for ATP with $K_a = 0.7\,\mu M$ (Keppens & De Wulf, 1985). (b) The cells were challenged for 2 min with glucagon (20 nM) and then for 2 min with ADPβS (●; solid line). The dashed line represents the curve for ATP with $K_i = 15\,\mu M$ (Keppens *et al.*, 1991). Results are expressed as percentage effect and are means ± s.e.mean. ($n = 4$). Lines drawn for the activation of phosphorylase are computer generated using the fitting facilities of the FigP programme or hand drawn for the inhibition of cyclic AMP increases.

crease the K_a value of phosphorylase activation after ATP (Keppens & De Wulf, 1991 and Figure 5b) about 2–3 fold. However, when used against ADPβS (Figure 5a), the activation of the enzyme was almost completely abolished.

In a further series of experiments, we examined the binding of ADPβ[^{35}S] to liver plasma membranes. Figure 6 illustrates the time-dependent association pattern for three different concentrations of ADPβ[^{35}S] and the dissociation of bound ligand induced by the addition of an excess of ADP. The association, fitting a first order equation, is rapid and an apparent equilibrium is reached in about 2 min. From the

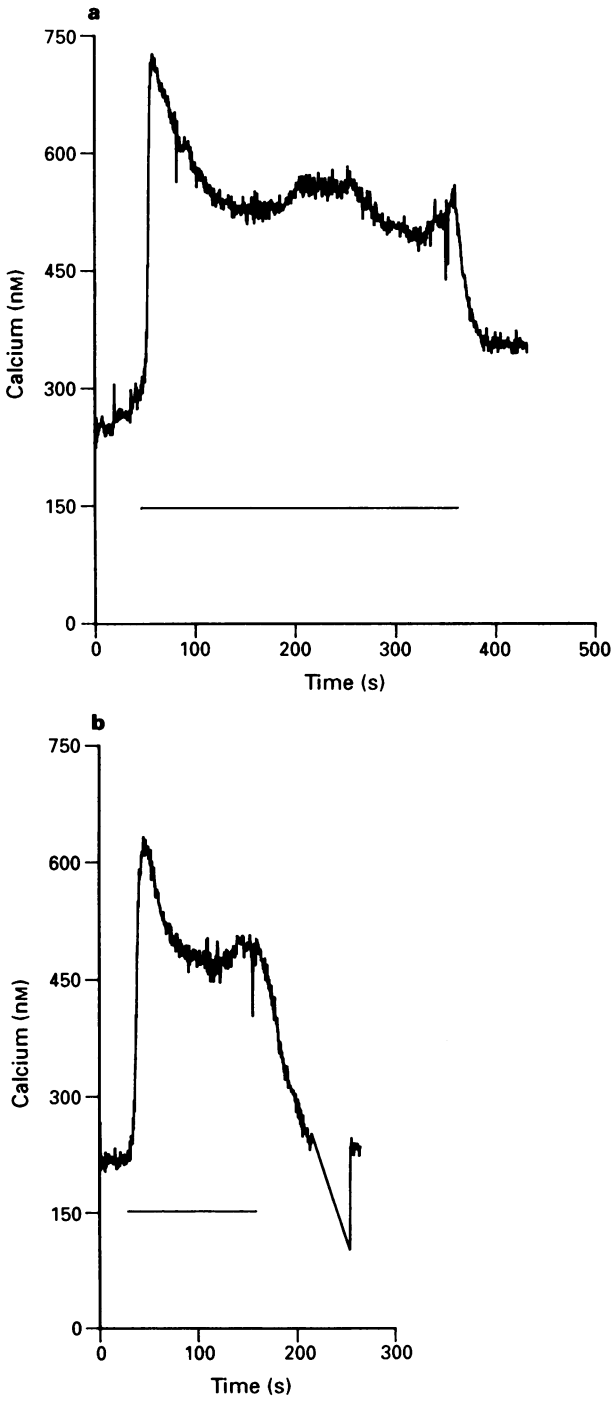

Figure 3 Increase of intracellular Ca^{2+}after adenosine 5'-[β-thio]diphosphate (ADPβS) and ATP in single hepatocytes loaded with Fura 2. A single hepatocyte was loaded with Fura 2 and superfused with buffer containing 2 μM ATP (a) or 2 μM ADPβS (b) during the time period indicated by the horizontal bar. Calcium concentrations were calculated as described in Methods.

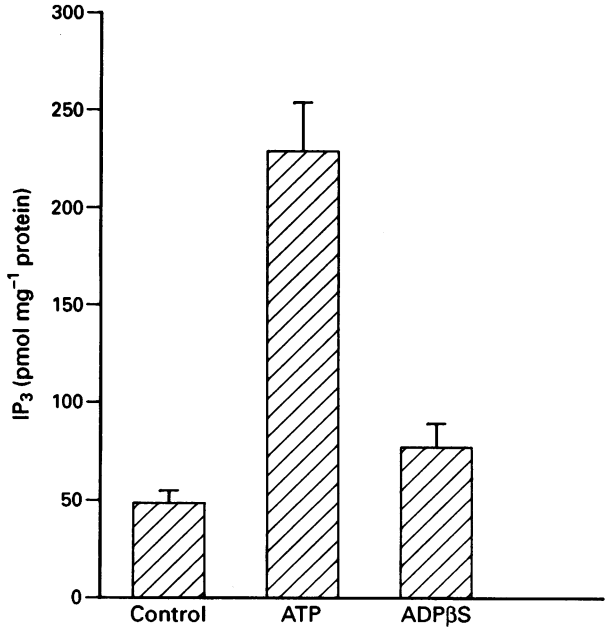

Figure 4 Effects of adenosine 5'-[β-thio]diphosphate (ADPβS) and ATP on inositol-(1,4,5)-trisphosphate (IP_3)-levels in hepatocytes. Hepatocytes were preincubated for 20 min at 37°C with 10 mM glucose. They were then challenged with 0.1 mM ADPβS or 0.1 mM ATP. Samples for IP_3-assay were taken 5 s later. Data shown are the means ± s.e.mean of three independent experiments, each done in triplicate. For ADPβS, the *t* test of independent data gave a *P* value of 0.05.

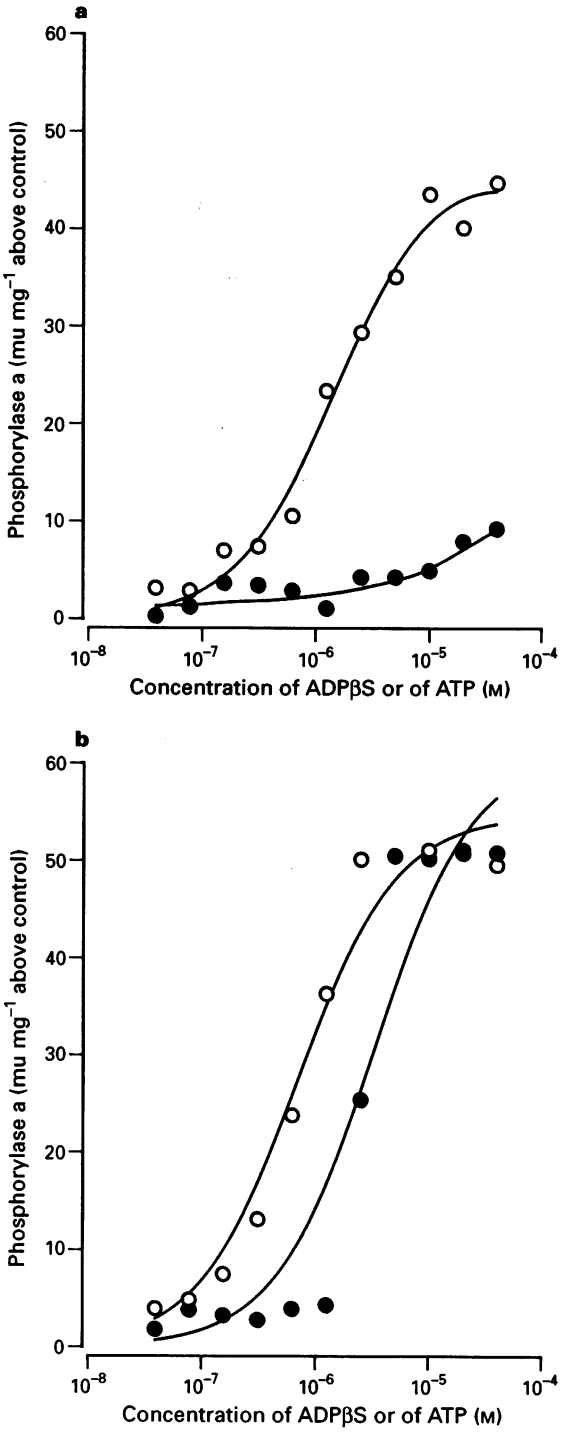

Figure 5 Effect of phorbol treatment of hepatocytes on the activation of glycogen phosphorylase by adenosine 5'-[β-thio]diphosphate (ADPβS) or ATP. Hepatocytes were first preincubated for 20 min at 37°C with 10 mM glucose and for another 10 min with either 1% dimethyl-sulphoxide (○) or phorbol myristic acetate (1.6 μM) dissolved in DMSO (●). Afterwards, increasing concentrations of ADPβS (a) or ATP (b) were added. Control values of phosphorylase were 9.64 ± 1.59 mu mg^{-1} of protein. Results shown are the mean of three independent experiments. Lines drawn are computer generated, using the fitting facilities of the FigP programme.

binding date, a k_{+1} (association rate constant) of 7.5 μM^{-1} min^{-1} has been calculated. The dissociation is also rapid with a k_{-1} (dissociation rate constant) of 3.5 min^{-1}. The ratio of both kinetic constants yields a dissociation constant K_D of about 0.5 μM. However, the dissociation is not complete and the reversibility of the binding but not the dissociation rate decreases with time. For the direct determination of the dissociation constant, different concentrations of the radioligand were incubated with plasma membranes for 1 min. We limited the incubation time to 1 min since at that time equilibrium is nearly obtained and reversibility is still more than 60% (Figure 6). Figure 7 shows the dose-dependent binding of ADPβ[^{35}S] to liver plasma membranes and the inset represents the Scatchard plot of the binding data. There is one major binding component with a B_{max} value of 51 pmol mg^{-1} of protein and with a dissociation constant K_D of 0.7 μM, a value close to the one deduced from the kinetic experiments. There is also a second but minor binding component with a B_{max} of 2.3 pmol mg^{-1} of protein and a dissociation constant in the nanomolar region (7 nM).

Discussion

ADPβS, the nonhydrolysable analogue of ADP, has been shown to elicit a P_{2Y}-purinoceptor-mediated response in plasma membranes of turkey erythrocytes. The agonist is even more powerful than ATP in stimulating inositol phosphate formation via a G-protein-dependent-phospholipase C (Boyer *et al.*, 1989; Berrie *et al.*, 1989) and could be used as a desensitizing agent for the enzyme (Martin & Harden, 1989). Moreover, the radioactive compound ADPβ[^{35}S] radiolabelled specifically the P_{2Y}-purinoceptor binding sites (Cooper *et al.*, 1989). It was therefore of interest to investigate the usefulness of ADPβS and ADPβ[^{35}S] as, respectively, a P_{2Y}-purinoceptor agent and radioligand for rat liver.

Firstly, the biological effects of ADPβS were compared with those of ATP. The results lead to distinct conclusions. The kinetics and dose-dependencies of the activation of glycogen phosphorylase (Figure 1 and Figure 2a) and of the inhibition of the increase in cyclic AMP levels after glucagon (Figure 1 and Figure 2b) as well as the increase in cytosolic Ca^{2+} (Figure 3) are almost identical for both agonists. These data could be interpreted to mean that binding occurs at the same P_{2Y}-type of purinoceptor. This conclusion is however

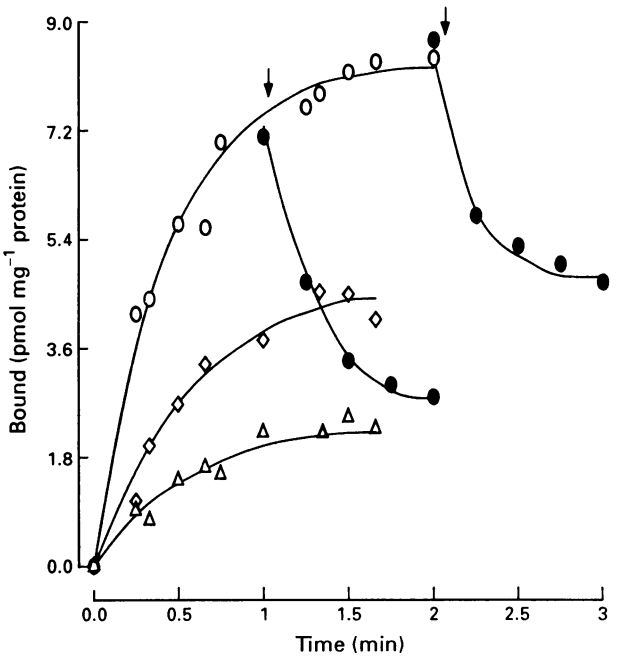

Figure 6 Association-dissociation pattern for the binding of adenosine 5'-[β-[^{35}S]thio]diphosphate (ADPβ[^{35}S]) to rat liver plasma membranes. Plasma membranes (0.6 mg of protein ml^{-1}) were incubated with 130 nM (○), 65 nM (◇) or 32 nM (△) ADPβ[^{35}S]. An excess of ADP (0.1 mM) was then added at the indicated times (arrows). Specific binding is plotted as a function of time. Values shown are means ($n = 3$ for the association and $n = 2$ for the dissociation).

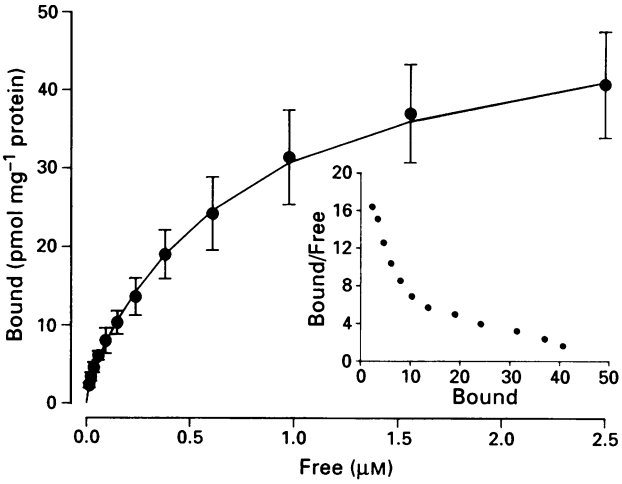

Figure 7 Dose-dependency of specific adenosine 5'-[β-[^{35}S]thio]-diphosphate (ADPβ[^{35}S]) binding to rat liver plasma membranes. Rat liver plasma membranes (0.6 mg of protein ml^{-1}) were incubated with the indicated concentrations of APDβ[^{35}S]. Specific binding was measured after 1 min; the values are given as means ± s.e.mean ($n = 5$). The inset shows the Scatchard plot of the binding data.

not consistent with other results. Indeed, in contrast to ATP but similar to 2MeSATP (Keppens & De Wulf, 1991; see also Introduction), which is also a powerful glycogenolytic agent, we found that ADPβS generated IP_3 only very moderately, even at very high concentrations, and that PMA almost completely counteracted its glycogenolytic effect. This either indicates that ADPβS interacts with poor affinity to the site linked to the activation of phospholipase C, or, alternatively, does interact with this site but produces only a partial effect. In both cases, a small increase (possibly sufficient for full activation of phosphorylase) is observed. In the latter case, and assuming that the affinity of ADPβS is about 0.7 μM (see later), one would expect ADPβS to inhibit the rise of IP_3 normally observed after ATP. We did not observe such an inhibition, suggesting that ADPβS, as also proposed for 2MeSATP (Keppens & De Wulf, 1991) binds to other P_2-purinoceptors, different from the classical P_{2Y}-receptor. It is however very unlikely that ADPβS and 2MeSATP activate the same binding site: indeed, in contrast to ADPβS (Figure 1b,2b), 2MeSATP is not able to suppress the cyclic AMP-rise after glucagon (Keppens & De Wulf, 1991).

The overall conclusion from our results with ADPβS is that this agonist may bind to a third type of P_2-receptor in rat liver parenchymal cells. This is in contrast with the situation in turkey erythrocytes in which ADPβS, like 2MeSATP, is a very powerful P_{2Y}-purinoceptor agonist (Boyer *et al.*, 1989; Berrie *et al.*, 1989; Martin & Harden, 1989). Apparently, the liver plasma membrane contains a complex mixture of P_2-purinoceptors with different affinities for different analogues. Alternatively, other transducing mechanisms could be triggered.

Okajima *et al.* (1987) demonstrated that for a series of purinoceptor agents, all activating phosphorylase and inhibiting the cyclic AMP-rise after forskolin treatment, two groups of agonists could be distinguished. One group, including GTP and ADP, exhibits similar abilities to that of ATP in raising inositolphosphate levels, whereas a second group, including α,β-methylene-ATP and β,γ-methylene-ATP affects only slightly inositol lipid metabolism. According to this classification, ADPβS would then belong to the second group of agonists.

The technique of recording Ca^{2+}-concentrations in single cells allows detection of differences at the cellular level. The fact that some cells, although sensitive to ATP, did not respond to ADPβS, could indicate that not all hepatocytes possess ADPβS-binding sites. Dixon *et al.* (1990) found differences between ATP and ADP in mobilizing intracellular Ca^{2+} in single cells. These authors obtained oscillations with clearly different frequencies for ATP and ADP and suggested the presence of two Ca^{2+}-mobilizing purinoceptors.

In the first part of this study, we have tentatively concluded that there are specific binding site(s) for ADPβS on rat hepatocytes. The question then arose whether ADPβ[^{35}S] could be used for further characterization of these site(s). From Figure 6 it is clear that although the binding is not fully reversible, the radioligand rapidly and specifically interacts with the liver plasma membrane receptor(s). A dissociation constant of about 0.7 μM could be derived from the kinetic experiments as well as from the equilibrium binding data (Figure 7). This binding component exhibits a K_D-value very close to the half-maximal activation of phosphorylase ($K_a = 1$ μM) by ADPβS and may therefore mediate the glycogenolytic effect of the agonist. The role for the minor binding component with a K_D of 7 nM, derived from the equilibrium binding study and representing about 5% of the total binding, is not obvious since no biological effect of ADPβS is observed at or below that concentration.

ADPβ[^{35}S] thus seems to be a suitable radioligand for detecting the binding sites mediating the biological effects of ADPβS in rat liver cells, bearing in mind that these sites differ from those used by ATP as well as by 2MeSATP.

This work was supported by the Belgian F.G.W.O. We thank G. Cumps for skillful technical assistance and M. Coppens for secretarial help.

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(Received July 15, 1992
Revised October 16, 1992
Accepted October 22, 1992)