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# BINDING OF THE RADIOLIGAND [$^{35}$S]ADENOSINE 5′-*O*-(2-THIODIPHOSPHATE) AND INTRACELLULAR CALCIUM RESPONSE IN RAT LIVER PARENCHYMAL CELLS

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**Abstract**—The use of the radioligand [$^{35}$S]adenosine 5′-*O*-(2-thiodiphosphate) (ADP$\beta^{35}$S) for the determination of $P_{2y}$-purinoceptors on turkey erythrocyte membranes has recently been described. In the present study, we were able to demonstrate specific binding of this radioligand in intact rat liver parenchymal cells. Within 10 min a thermodynamic equilibrium was obtained which lasted for 25 min with a subsequent decline. Displacement studies with several nucleotides were performed yielding $K_i$ values of 1.5 ± 0.47 μM for UTP, 1.8 ± 0.35 μM for adenosine 5′-*O*-(2-thiodiphosphate) (ADPβS), 31 ± 6.2 μM for ATP and 35 ± 6.1 μM for GTP. In addition, we showed that ADP$\beta^{35}$S is highly resistant to degradation by ecto-nucleotidases, with only 14.5 ± 1.4% of total ADP$\beta^{35}$S present being degraded after 1 hr, and that the binding of ADP$\beta^{35}$S to its binding sites was modulated by EDTA. The $K_i$ value of ATP shifted to 8.1 ± 1.2 μM upon the addition of 1 mM EDTA to the incubation medium. In these rat liver parenchymal cells all nucleotides promoted calcium entry in a dose-dependent manner with $EC_{50}$ values of 3.5 ± 0.22 μM for UTP, 20.7 ± 3.1 μM for ATP, 38.3 ± 6.4 μM for ADPβS and 73.6 ± 13.7 μM for GTP, with GTP being a partial agonist. Based on the data derived from the present study we discuss the possible correlation between binding and functional experiments and conclude that the described receptor resembles most closely the $P_{2u}$-purinoceptor and/or "nucleotide receptor", in that UTP is at least as active as ATP.

ATP can act as a non-adrenergic, non-cholinergic neurotransmitter or as a humoral modulator that exerts its actions in micromolar concentrations via the $P_2$-purinergic receptors [1]. Initially two subclasses, $P_{2x}$ and $P_{2y}$, were distinguished on the basis of rank order of potency of various ATP-analogues in functional pharmacological studies [2]. More recently other subtypes were described, amongst which the $P_{2u}$-purinoceptor [3] and/or the general "nucleotide receptor" [4], that are not only sensitive to purine nucleotides but to pyrimidine nucleotides as well. In 1989, Cooper *et al.* [5] first described a radioligand binding assay, using [$^{35}$S]-adenosine 5′-*O*-(2-thiodiphosphate) (ADP$\beta^{35}$S‡), for quantification of the $P_{2y}$-receptor on turkey erythrocyte membranes. In addition, they showed that this purinergic receptor was coupled to a guanine nucleotide binding protein-linked phospholipase C. Various authors have shown that there is a correlation between the phospholipase C activity in a cell and its intracellular calcium concentration ($[Ca^{2+}]_i$) [6]. Others have described a phospholipase C-independent, but extracellular calcium-dependent response [7]. Because we also observed a rise in the $[Ca^{2+}]_i$ in liver parenchymal cells when stimulated with ATP, we set out to investigate the $P_2$-purinoceptor mediating this effect on these cells. In the present report, we aim to show that there may be a correlation between binding of the radioligand ADP$\beta^{35}$S to $P_2$-purinoceptors present on rat liver parenchymal cells and the $[Ca^{2+}]_i$ in these cells. We also conclude that the observed calcium response is dependent on the presence of extracellular calcium and hence is the result of calcium entry rather than of calcium mobilization.

## MATERIALS AND METHODS

*Materials.* ATP, GTP and Tris were purchased from Aldrich Chemie (Brussels, Belgium). Adenosine 5′-*O*-(2-thiodiphosphate) (ADPβS), EDTA, ethylene glycol bis(2-aminoethylether)-*N,N,N′,N′*-tetra acetic acid (EGTA) and Quin-2 were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). ADP$\beta^{35}$S (initial specific activity of approx. 38.5 TBq/mmol) was purchased from Du Pont-New England Nuclear (Herts, U.K.). Emulsifier Safe scintillation fluid was from Packard (Amstelveen, The Netherlands). UTP, collagenase (EC 3.4.24.3) and 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid (Hepes) were from Boehringer Mannheim (Mannheim, Germany). Dimethyl sulphoxide (DMSO) was from Baker (Deventer, The Netherlands) and charcoal (particle size 4–7 μm) was from Serva Feinbiochemica (Heidelberg, Germany).

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‡ Abbreviations: ADP$\beta^{35}$S, [$^{35}$S]adenosine 5′-*O*-(2-thiodiphosphate); ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); $[Ca^{2+}]_i$, intracellular free calcium concentration; EGTA, ethylene glycol bis(2-aminoethylether)-*N,N,N′,N′*-tetra acetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid; DMSO, dimethyl sulphoxide; $[Ca^{2+}]_i^0$, intracellular free calcium concentration under resting conditions; [$^{35}$S]ATPαS, [$^{35}$S]adenosine 5′-*O*-(1-thiotriphosphate).

The 12.5 mL polypropylene incubation tubes were from Greiner bv (Alphen a.d. Rijn, The Netherlands) and the GF/C glass fibre filters were from Whatman Ltd (Maidstone, U.K.). Male SPF Wistar rats of about 250 g were obtained from the Sylvius Laboratories, Leiden University (Leiden, The Netherlands). All other chemicals used were of commercial grade. The water used was doubly glass distilled.

*Isolation of rat liver parenchymal cells.* Liver parenchymal cells were isolated by collagenase perfusion and differential centrifugation from rats that were fed *ad lib.*, as described previously [8]. Generally, over 95% of the isolated liver parenchymal cells were viable, as determined by a trypan blue exclusion test. This viability was maintained throughout the experiments by the use of buffers saturated with carbogen. After isolation, the cells were kept at 0° in a buffer containing 137 mM NaCl, 5.4 mM KCl, 0.81 mM $MgSO_4$, 0.84 mM $Na_2HPO_4$, 0.44 mM $KH_2PO_4$, 19.2 mM Hepes, 5.56 mM glucose, 1.3 mM $CaCl_2$ which was saturated with carbogen gas (95% $O_2$ + 5% $CO_2$) and adjusted to pH 7.4 after saturation with carbogen.

*Determination of the dephosphorylation of ADP$\beta^{35}$S by liver parenchymal cells.* The freshly isolated liver parenchymal cells were washed three times with the incubation medium (10 mM Hepes, 142 mM NaCl, 6.7 mM KCl, pH 7.4 which was saturated with carbogen) by centrifugation at 25 *g* for 2 min at ambient temperature. The incubation was then performed in 12.5 mL polypropylene tubes, containing 0.38–0.40 nM ADP$\beta^{35}$S in a total volume of 200 $\mu$L. The incubation was started with the addition of 50 $\mu$L of a cell suspension containing $0.15 \times 10^6$ cells. The cells were incubated under carbogen at 37° for 15, 30 or 60 min. The incubation was stopped by the addition of 400 $\mu$L of a suspension of charcoal in 0.1 M HCl (4 g/100 mL), followed by centrifugation at 6500 *g* for 2 min. An aliquot of 200 $\mu$L of the supernatant containing the released inorganic thiophosphate was counted in 3.5 mL Emulsifier Safe. The experiments were repeated with either 1 mM EDTA or 10 $\mu$M ADP$\beta$S in the incubation medium.

*Radioligand binding experiments with freshly isolated liver parenchymal cells.* The freshly isolated liver parenchymal cells were washed three times with the incubation medium (10 mM Hepes, 142 mM NaCl, 6.7 mM KCl, pH 7.4 which was saturated with carbogen) by centrifugation at 25 *g* for 2 min at ambient temperature. The determination of the equilibrium conditions was performed in a 12.5 mL polypropylene tube containing a mixture of 425 $\mu$L 2.0 nM ADP$\beta^{35}$S, 1700 $\mu$L incubation medium and 2125 $\mu$L of a cell suspension containing $30 \times 10^3$ cells/50 $\mu$L. The incubation (at 37° under carbogen) was started with the addition of the ADP$\beta^{35}$S and an aliquot of 200 $\mu$L was taken at various time intervals. The aliquot was quenched in 9 mL ice-cold washing buffer (20 mM Tris, 145 mM NaCl, pH 7.4 at 0°), immediately followed by filtration under reduced pressure (60 kPa) over GF/C filters presoaked in washing buffer. The tubes and filters were rinsed with another 9 mL washing buffer and the filters were dried for 45 min at 75°. The radioactivity on the filters was counted after 2 hr of extraction in 3.5 mL Emulsifier Safe. The displacement studies were performed in 12.5 mL polypropylene tubes, containing 0.20–0.25 nM ADP$\beta^{35}$S and varying concentrations of competitor in the incubation buffer in a total volume of 200 $\mu$L. The incubation was started with the addition of 50 $\mu$L of a cell suspension containing $30 \times 10^3$ cells. The cells were incubated under carbogen for 30 min at 37°. The incubation was stopped with the addition of 9 mL ice-cold washing buffer (20 mM Tris, 145 mM NaCl, pH 7.4 at 0°), immediately followed by filtration under reduced pressure (60 kPa) over GF/C filters presoaked in washing buffer. The tubes and filters were rinsed with another 9 mL washing buffer and the filters were dried for 45 min at 75°. The radioactivity on the filters was counted after 2 hr of extraction in 3.5 mL Emulsifier Safe.

*Determination of the free intracellular calcium concentration in liver parenchymal cells.* The free intracellular calcium concentration was determined as described earlier [8]. Liver parenchymal cells were preloaded with Quin-2 and resuspended in 3 mL incubation buffer containing 120 mM NaCl, 5.3 mM KCl, 4.2 mM $NaHCO_3$, 1.2 mM $KH_2PO_4$, 2.6 mM $CaCl_2$, 1.3 mM $MgSO_4$, 13.6 mM glucose, 20 mM Hepes, 0.2% bovine serum albumin (w/v), pH 7.4 at 37°, saturated with carbogen, at a cell density of $1 \times 10^6$ cells/mL. In the case of measurements in a calcium-free buffer the $CaCl_2$ was omitted from this buffer. The fluorescence ($\lambda_{ex}$ = 339 nm, $\lambda_{em}$ = 492 nm) was measured continuously for 2 min after the addition of either ATP, UTP, GTP or ADP$\beta$S. The experiments were ended with the subsequent addition of 3 $\mu$L 5 mM digitonin in DMSO/$H_2O$ (50/50 v/v) to determine the maximum fluorescence and 100 $\mu$L 500 mM EGTA/NaOH in water (pH 7) to determine the minimum fluorescence. $[Ca^{2+}]_i$ was calculated by interpolation between these last two values [9].

*Data analysis.* All radioligand binding experiments were performed in duplicate. Data presented here are means ± SEM of three separate experiments unless stated otherwise. The data were analysed as described by IJzerman *et al.* [10] with a non-linear curve-fitting program based on the law of mass action. The $K_i$ values were calculated from the $IC_{50}$ values following Cheng and Prusoff [11]: $K_i = IC_{50}/(1 + L^*/K_d^*))$, where $L^*$ is the radioligand concentration and $K_d^*$ is the radioligand equilibrium dissociation constant. Since $L^* \ll K_d^*$ in all binding experiments it follows that $K_i \simeq IC_{50}$ (see also Discussion). For the calcium measurements all experiments were performed in triplicate, unless stated otherwise, and typical experiments are represented in the figures. Data presented here are means ± SEM.

## RESULTS

### *Determination of the dephosphorylation of ADP$\beta^{35}$S by liver parenchymal cells*

Figure 1 shows that breakdown of ADP$\beta^{35}$S by liver parenchymal cells after a 60 min incubation was only 14.5 ± 1.4%. Breakdown of ADP$\beta^{35}$S by liver parenchymal cells was significantly reduced to

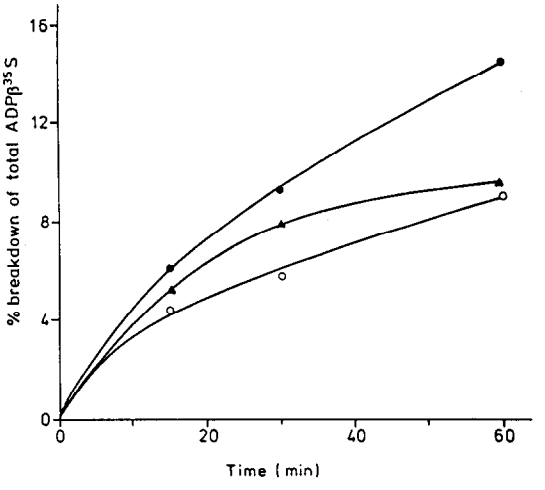


Fig. 1. Dephosphorylation of ADPβ[35]S in the presence of rat liver parenchymal cells. Data are presented as {(activity after incubation − activity before incubation)/(total activity added)} × 100%. Breakdown of ADPβ[35]S as percentage of the total amount of ADPβ[35]S present in the incubation medium without cells present did not exceed 1.5% after a 60 min incubation (N = 2). (●) 0.38–0.40 nM ADPβ[35]S; (▲) 0.38–0.40 nM ADPβ[35]S + 10 μM ADPβS; (○) 0.38–0.40 nM ADPβ[35]S + 1 mM EDTA.

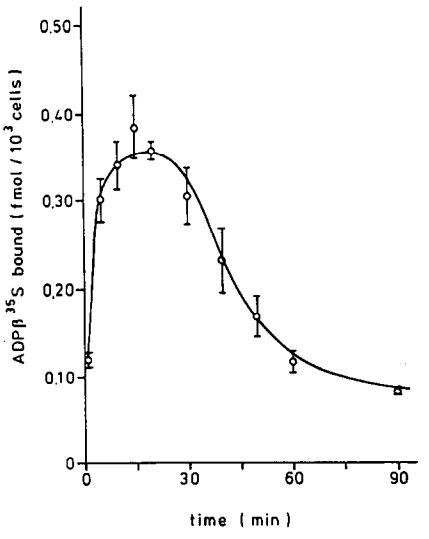


Fig. 2. Time-dependency for equilibrium of ADPβ[35]S binding on rat liver parenchymal cells. The cells were incubated with 0.20 nM ADPβ[35]S in a 10 mM Hepes, 142 mM NaCl, 6.7 mM KCl, pH 7.4, 37° buffer, that was saturated with carbogen, and aliquots were taken at various time intervals.

9.1 ± 1.4% (*t*-test, $P < 0.025$) by the addition of 1 mM EDTA to the incubation medium. A similar reduction was obtained by the addition of 10 μM ADPβS, the breakdown being significantly decreased to 9.7 ± 2.2% (*t*-test, $P < 0.025$).

*Determination of the equilibrium conditions for ADPβ[35]S binding on liver parenchymal cells*

Figure 2 shows the time-dependency of ADPβ[35]S binding to liver parenchymal cells. Within the first 10 min binding increases rapidly and then levels off to a plateau value of approx. 0.38 fmol/$10^3$ cells. The binding remains stable up to 35 min after the start of the experiment and then decreases until a value of 0.08 fmol/$10^3$ cells is reached. The shape of this curve precluded the determination of the kinetic parameters $k_{+1}$ (association rate constant) and $k_{-1}$ (dissociation rate constant).

*Radioligand binding experiments with freshly isolated liver parenchymal cells*

The affinities of several nucleotides for the ADPβ[35]S binding site on the rat liver parenchymal cell are shown in Table 1, with UTP being the most potent displacer ($K_i$ value is 1.5 ± 0.47 μM). The $K_i$ value of ADPβS (1.8 ± 0.35 μM) rules out the possibility to determine a saturation curve, necessary to obtain the total concentration of receptors ($B_{max}$). Under the experimental conditions the specific binding on liver parenchymal cells incubated without EDTA was 0.35 ± 0.05 fmol/$10^3$ cells, whereas the specific binding on liver parenchymal cells incubated with 1 mM EDTA was 0.84 ± 0.17 fmol/$10^3$ cells. This coincided with a shift in $K_i$ values for ATP from 31 ± 6.2 μM in the absence of EDTA to 8.1 ± 1.2 μM in the presence of 1 mM EDTA. The data for ATP are graphically represented in Fig. 3.

*Determination of the* $[Ca^{2+}]_i$ *in liver parenchymal cells*

Figure 4 shows representative examples of the experiments with the calcium-sensitive fluorescent probe Quin-2.

The experiments started with the determination of the intracellular free calcium concentration under resting conditions ($[Ca^{2+}]_i^0$) which was 140 ± 6.5 nM (N = 10). The initial peak in the fluorescence signal, and those upon the addition of either digitonin or EGTA, are due to the method and have no physiological relevance. The $[Ca^{2+}]_i$ tends to drop back to basal levels after 1 min after the addition of the nucleotide and all $[Ca^{2+}]_i$ were therefore determined 1 min after the addition of the nucleotide. $[Ca^{2+}]_i^0$ remained unchanged and no calcium response could be observed in the absence of extracellular calcium (results not shown). It should be noted that $[Ca^{2+}]_i$ higher than 3000 nM should be considered as approximations due to the use of Quin-2 as a fluorescent probe. The order of potency of the nucleotides, however, is not affected by this methodological problem.

The dose–response curves compiled from the experiments described above are represented in Fig. 5. The $EC_{50}$ values calculated from these curves are shown in Table 1. UTP was the most potent agonist with an $EC_{50}$ value of 3.5 ± 0.22 μM. The figure also shows that GTP is a partial agonist with an intrinsic activity of 0.45 relative to ATP. The shape of the dose–response curves is striking: a maximal effect is obtained at a concentration of the nucleotide approx.

Table 1. Effect of various nucleotides on ADP$\beta^{35}$S binding and $[Ca^{2+}]_i$ response

| Ligand | ADP$\beta^{35}$S-binding [EDTA] (mM) | $K_i$ ($\mu$M) | N | $[Ca^{2+}]_i$ response $EC_{50}$ ($\mu$M) | N |
|---|---|---|---|---|---|
| ADP$\beta$S | 0 | 1.8 ± 0.35 | 3 | 38.3 ± 6.4 | 3 |
| ATP | 0 | 31.0 ± 6.2 | 5 | 20.7 ± 3.1 | 3 |
| ATP | 1 | 8.1 ± 1.2 | 5 | | |
| GTP | 0 | 35.0 ± 6.1 | 3 | 73.6 ± 13.7 | 3 |
| UTP | 0 | 1.5 ± 0.47 | 3 | 3.5 ± 0.22 | 3 |

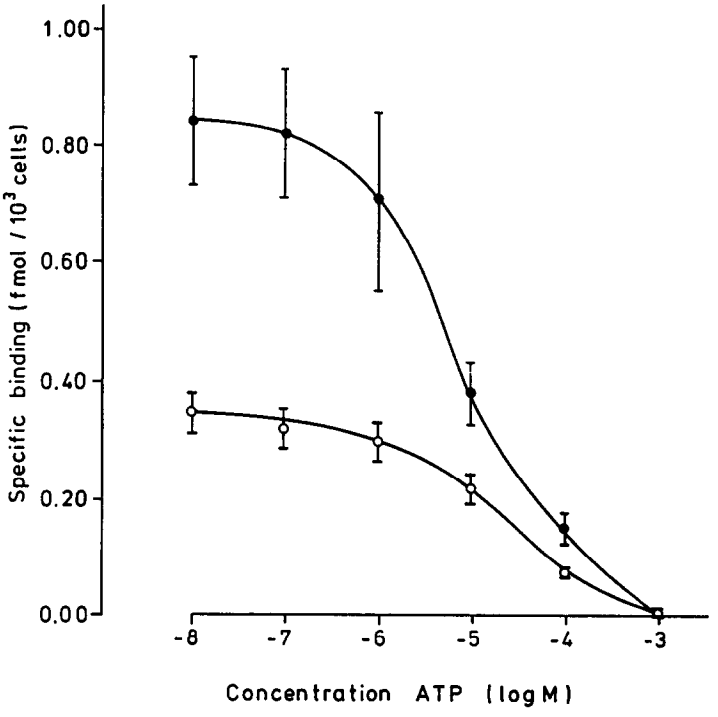


Fig. 3. Displacement of ADP$\beta^{35}$S by ATP on rat liver parenchymal cells in the presence or the absence of EDTA in the incubation medium. The cells were incubated with 0.20–0.25 nM ADP$\beta^{35}$S in a 10 mM Hepes, 142 mM NaCl, 6.7 mM KCl, pH 7.4, 37° buffer, that was saturated with carbogen, in a total volume of 200 $\mu$L and varying concentrations of the competitor ATP. (○) ADP$\beta^{35}$S, (●) ADP$\beta^{35}$S + 1 mM EDTA.

10 times higher than the initial concentration that marked the onset of the response. The shape of the ATP curve deviates somewhat from the other curves in that it is not as steep. This deviation may be the result of a biphasic response, an initial increase in $[Ca^{2+}]_i$ to approx. 600 nM and a subsequent rise to 5000 nM, as discussed later (*vide infra*).

## DISCUSSION

### *Radioligand binding experiments*

In 1986, Keppens and De Wulf [12] showed that the radioligand [$^{35}$S]adenosine 5′-*O*-(1-thiotriphosphate) ([$^{35}$S]ATP$\alpha$S) was able to bind $P_2$-purinoceptors and subsequently activate glycogen phosphorylase in a rat liver preparation. Rat liver parenchymal cells possess, like many other cells, ecto-nucleotidases on the outer surface membrane [13]. These ecto-nucleotidases are able to hydrolyse nucleoside (poly)phosphates, irrespective of the base moiety. Modification of the phosphodiester linkages with e.g. methylene groups or substitution of a phosphate group by a thiophosphate group usually induces resistance to this ecto-nucleotidase activity. A disadvantage of [$^{35}$S]ATP$\alpha$S is the liability to dephosphorylation of this nucleotide by these ecto-nucleotidases [14]. To overcome this problem we used the more stable nucleotide ADP$\beta^{35}$S. This radioligand was tested for its resistance to ecto-nucleotidase activity, because the lysis of the

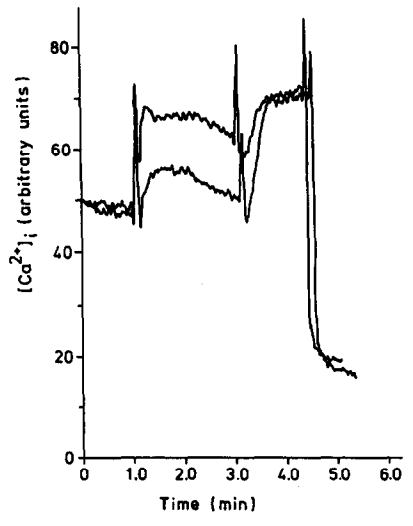


Fig. 4. Representative examples of the calcium response in liver parenchymal cells induced by 3 μM ATP and 3 μM ADPβS, respectively. The extracellular medium contained 2.6 mM $Ca^{2+}$. The maximum and minimum responses were elicited with 3 μL 5 mM digitonin and 100 μL 500 mM EGTA, respectively. The $[Ca^{2+}]_i^0$ was 140 ± 6.5 nM. The upper tracing was 3 μM ATP and the lower tracing was 3 μM ADPβS. The first spike indicates the addition of the nucleotide, the second spike indicates the addition of the digitonin and the third spike indicates the addition of the EGTA.

radioligand would obscure the effects observed at the receptor level, especially when using high concentrations of the radioligand ADPβ$^{35}$S or its non-radioactive analogue ADPβS. It was shown that the cumulative production of inorganic thiophosphate after 60 min due to breakdown of ADPβ$^{35}$S in the presence of liver parenchymal cells was only 14.5 ± 1.4% of the total amount of ADPβ$^{35}$S present. This is consistent with the findings of Cusack *et al.* [14] who showed that ADPβS is highly resistant to enzymatic degradation. The breakdown of ADPβ$^{35}$S could be attenuated by the addition of 1 mM EDTA to the incubation medium. This is consistent with the fact that ecto-nucleotidases are dependent on divalent metal ions, as shown by Lin and Russell [13]. The cumulative production of inorganic thiophosphate was not proportional to the incubation time. Since $K_m$ values of ecto-nucleotidases range between 20 and 100 μM, enzymatic breakdown of ADPβ$^{35}$S at a concentration of 0.4 nM is supposed to be substrate-limited [13]. Because ADPβ$^{35}$S was not degraded for more than 14.5% within 1 hr this could not be the major reason for the deviation from linearity. Moreover, the addition of 10 μM non-radioactive ADPβS also attenuated the enzymatic breakdown of ADPβ$^{35}$S. This is suggestive of a mechanism of inhibition where the hydrolysis products accumulate in the active site of the enzyme. However, since this was not the aim of our study we did not investigate this mechanism further.

Having established the usefulness and stability-characteristics of ADPβ$^{35}$S, we investigated the

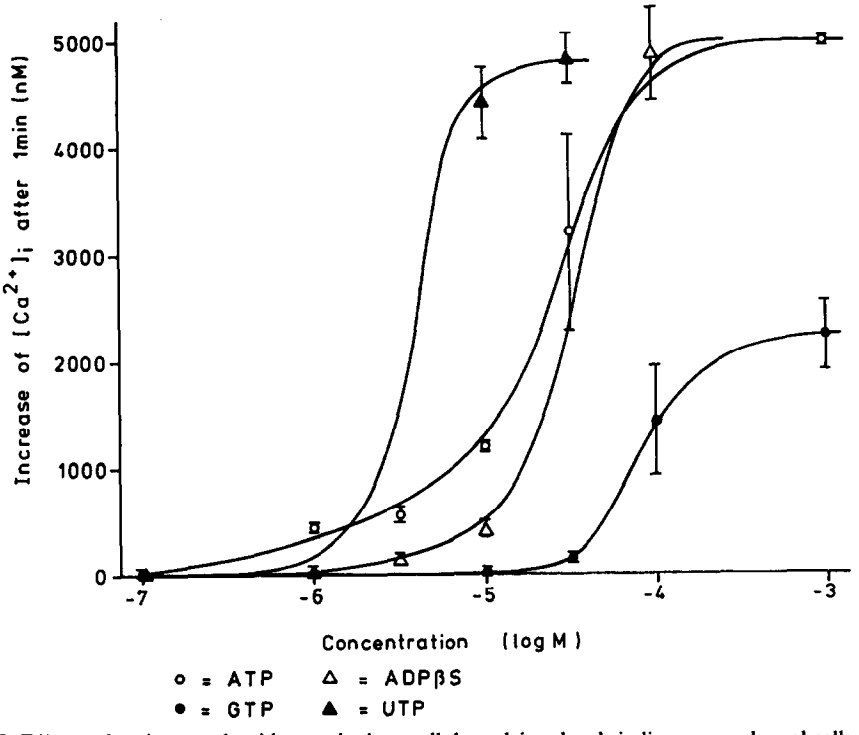


Fig. 5. Effects of various nucleotides on the intracellular calcium levels in liver parenchymal cells 1 min after the addition of the nucleotide. UTP (▲) is the most potent agonist, and maximum stimulation could be obtained at a concentration of 30 μM. GTP (●) seems to be a partial agonist, since no maximal response could be obtained at the highest concentration used.

receptor binding-characteristics of this radioligand. From the determination of the equilibrium conditions (Fig. 2), a standard equilibration time of 30 min was chosen. The reason for the decrease in binding after 35 min is unknown. High affinity binding sites were demonstrated on the freshly isolated rat liver parenchymal cells. The displacement studies yielded the rank order of affinity UTP = ADPβS > ATP = GTP. This potency order is indicative of a $P_{2u}$-purinoceptor or a "nucleotide receptor", because UTP is the most potent ligand. O'Connor *et al.* [4] noticed ADPβS to be only marginally effective on the "nucleotide receptor" in rat and piglet aorta preparations, whereas ADPβS is a rather potent ligand in our test system. There is a notable difference in affinity of ADPβ$^{35}$S for turkey erythrocyte membranes and rat liver parenchymal cells. On turkey erythrocyte membranes the $K_d$ value of the radioligand was 8.5 ± 0.5 nM, in good agreement with the $K_i$ value of unlabelled ADPβS of 33.2 ± 2.0 nM, as determined by Cooper *et al.* [5], whereas on rat liver parenchymal cells the $K_i$ value was 1.8 ± 0.35 μM. This high $K_i$ value unfortunately precluded the accurate determination of the $K_d$ value of ADPβ$^{35}$S on our cell preparation. The difference in affinity may be attributed to the species difference; an avian vs a mammalian $P_2$-purinoceptor. Another possibility is that ADPβ$^{35}$S does not label the same binding site in turkey erythrocytes and on rat liver parenchymal cells, which could be substantiated by the fact that the potency order for the nucleotides tested on turkey erythrocytes adheres to the definition of the $P_{2y}$-purinoceptor [5], whereas on rat liver parenchymal cells UTP is more potent than ATP.

Another feature of the radioligand binding studies is the dependency of the specific binding and the $K_i$ value of ATP on the presence of EDTA. The specific binding increases and the $K_i$ value decreases upon the addition of EDTA to the incubation medium. The increase in specific binding may be caused by either an increase in the affinity of the radioligand ($K_d$) or an increase in the total number of binding sites ($B_{max}$). Cooper *et al.* [5] noticed that omission of $Mg^{2+}$ from the buffer increased binding of ADPβ$^{35}$S to the receptor and this is in accordance with our experiments with EDTA, that could chelate residual $Mg^{2+}$ ions.

*Determination of the intracellular free calcium concentration*

The activation of glycogen phosphorylase by ATPαS as shown by Keppens and De Wulf [12], was attributed by Charest *et al.* [15] to an increase in the intracellular inositol 1,4,5-trisphosphate concentration and the mobilization of intracellular calcium. The rank order of potency as derived from our calcium measurements is UTP > ATP = ADPβS > GTP. The $P_2$-purinoceptor on rat hepatocytes described by Charest *et al.* [15] appears to be of another subtype since the $EC_{50}$ for the stimulation of $Ca^{2+}$ mobilization by ATP was 0.8 μM whereas in the present study the $EC_{50}$ for $Ca^{2+}$ entry was 20.7 μM. Also the maximal response was different: the maximal $[Ca^{2+}]_i$ after $Ca^{2+}$ mobilization was 625 nM whereas the maximal $[Ca^{2+}]_i$ after $Ca^{2+}$ entry exceeded 4000 nM. When studying Fig. 5 in detail the co-existence of both subtypes could be suggested as only ATP shows a moderate increase at low ATP concentrations and a large increase at high ATP concentrations, where the other nucleotides only elicit a large response. The fact that no response could be evoked when the extracellular calcium was omitted, however, argues against the co-existence of these two receptor subtypes, since the receptor described by Charest *et al.* [15] seems to be independent of the presence of extracellular calcium. A reason for these apparent differences is not known. The $[Ca^{2+}]_i^0$ remained unchanged when the extracellular calcium was omitted and this indicates that the intracellular calcium stores were not depleted. The depletion of these calcium stores could have provided an alternative explanation for the absence of a calcium response in the absence of extracellular calcium.

The steep dose–response curves in Fig. 5 are indicative of a non-linear coupling between receptor occupation and effect, i.e. an "all-or-none" response comprising a threshold; a substantial fraction of the total receptor population has to be occupied before a response can be elicited. This feature is in line with the ligand-gated $Ca^{2+}$-channel type accommodating large inward currents, as described by Benham [16]. The order of potency for the activation of $Ca^{2+}$-influx favours the classification of this receptor subtype as a $P_{2u}$-purinoceptor [3] and/or a "nucleotide receptor" [4].

*Correlation between the two types of experiments*

The $EC_{50}$ values and the $K_i$ values for the experiments with UTP, ATP and GTP, as stated in Table 1, are all in the micromolar range and seem to be in reasonable agreement, suggesting a correlation between ADPβ$^{35}$S binding and calcium response. The $EC_{50}$ value for ADPβS, however, is approx. 20 times its $K_i$ value which appears to contradict a possible correlation. Due to the high ecto-nucleotidase activity of rat liver parenchymal cells [13] it is likely that the $K_i$ values found for the labile nucleotides UTP, ATP and GTP are an overestimation of the real values, because of the relatively long incubation period of 30 min relative to the 1 min incubation period for the calcium measurements. This would yield "corrected" $K_i$ values for these nucleotides that are essentially lower than their corresponding $EC_{50}$ values, as observed for the stable nucleotide ADPβS. The increased affinity of ATP upon the addition of EDTA (decreasing hydrolysis) in the radioligand binding experiments supports this reasoning (see Table 1). Because of the lack of a specific ecto-nucleotidase inhibitor it was not possible to investigate the effect of the ecto-nucleotidase activity on the $K_i$ values in detail. In view of the established differences between the $K_i$ and $EC_{50}$ values (up to 20-fold) the threshold for the nucleotides, as discussed earlier, may require as much as 90–95% receptor occupation. Thus, the use of the relatively stable nucleotide ADPβS appears to shed light on the coupling between receptor occupation and activation.

*Conclusion*

The radioligand ADP$\beta^{35}$S is suitable for the quantification of specific binding sites present on rat liver parenchymal cells, because it is highly resistant to enzymatic degradation by ecto-nucleotidases, it has a high specific activity and a relatively high affinity for the receptor. The binding of ADP$\beta^{35}$S to its binding sites is modulated by EDTA. Upon activation of hepatic $P_2$-purinoceptors by ADP$\beta$S and other nucleotides the $[Ca^{2+}]_i$ is raised through influx of extracellular calcium. Although not unambiguously proven, the ADP$\beta^{35}$S binding sites are likely to be identical to these $P_2$-purinoceptors. Since both the radioligand binding studies and the calcium measurements showed UTP to be at least equipotent with ATP we conclude that this $P_2$-purinoceptor on rat liver parenchymal cells is best described as a $P_{2u}$-purinoceptor and/or "nucleotide receptor" that mediates $Ca^{2+}$ entry into these cells.

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