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BINDING OF [^{35}S]ADENOSINE 5′-*O*-(2-THIODIPHOSPHATE) TO ENDOTHELIAL CELLS IN CULTURE

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Abstract—We have investigated the binding of [^{35}S]adenosine 5′-*O*-(2-thiodiphosphate) ([^{35}S]ADPβS) to intact cultured bovine aortic endothelial cells which have been previously shown to co-express P_{2y} and P_{2u} purinoceptors and to bovine adrenal medulla endothelial cells which solely possess P_{2u} purinoceptors. ADPβS has been shown to stimulate phospholipase C activity in these cells via the P_{2y} purinoceptor and does not interact with the P_{2u} purinoceptor. We describe a simple equilibrium binding procedure designed for the study of low affinity agonists and compare these results with those obtained by separation of bound and free by filtration. Saturation analysis of equilibrium binding data revealed two sites for ADPβS binding; one with $K_D = 3.3 \times 10^{-8}$ M, $B_{max} = 32$ pmol/mg protein; and the other with $K_D = 4.3 \times 10^{-6}$ and $B_{max} = 2155$ pmol/mg protein. Use of filtration did not significantly alter the K_D of either of these sites, nor the B_{max} of the high affinity site, but reduced the B_{max} of the low affinity site by more than 95%. The rank order of agonist potency for competing for [^{35}S]ADPβS binding indicated that most of this was to non-P_{2y} purinoceptor sites as β,γ-methylene ATP, a P_{2x} purinoceptor agonist, was more potent than 2-methylthio ATP, a P_{2y} purinoceptor agonist. Binding was also carried out in the presence of β,γ-methylene ATP, in an attempt to reduce non-P_{2y} purinoceptor binding and produced similar results. Specific [^{35}S]ADPβS binding sites were also found in bovine adrenal medulla endothelial cells which do not possess P_{2y} purinoceptors. These results indicate that [^{35}S]ADPβS was able to bind to endothelial cells from different parts of the vasculature but that the ligand can only be considered suitable for investigation of P_{2y} purinoceptors on mammalian cells when specific conditions are designed to reduce the large amount of non-receptor binding.

Key words: endothelial cells; P_{2y} purinoceptors; radioligand binding studies; [^{35}S]adenosine 5′-*O*-(2-thiodiphosphate)

The widespread importance of cell surface receptors for ATP (the P_2 purinoceptors) has been recognized for a number of years (for instance see Ref. 1, for recent review). Definitive pharmacological characterization of these receptors has been hampered by a lack of specific antagonists and radioligand binding assays and only recently has it become possible to study the molecular nature of this class of receptors with the cloning of the P_{2y} and P_{2u} purinoceptors [2–4]. Because of the absence of selective antagonists, binding studies on P_2 purinoceptors have been carried out using radiolabelled agonists. Rat urinary bladder and vas deferens membranes have been labelled by [^{3}H]α,β-methylene ATP, with pharmacology consistent with the presence of P_{2x} purinoceptors [5–7]. Analogues of ATP have been used to attempt binding to P_{2y} purinoceptors. For instance, in heart sarcolemma membranes, [^{35}S]ATPγS‡ labels two apparent affinity sites [8], while [^{35}S]ATPαS has been used to study purinoceptors from hepatocytes of man, rat and guinea pig [9–11]. Binding of these analogues, however, was compromised because they may not discriminate between different purinoceptors and in some cases binding was not saturable and may be unrelated to the receptor. Successful studies have been carried out on turkey erythrocyte membranes using [^{35}S]ADPβS as radioligand [12]. These avian erythrocytes have previously been shown to express a P_{2y} purinoceptor linked to the activation of PLC [13, 14]. The advantages of ADPβS are that it shows selectivity for P_{2y} purinoceptors, it is an analogue of ADP and may not bind to non-receptor sites for ATP, the sulphur substitution on the beta phosphate renders it relatively resistant to ectonucleotidase activity and it is available at high specific activity. [^{35}S]ADPβS binds to turkey erythrocyte membranes with pharmacology characteristic of a P_{2y} purinoceptor and with dependence on guanine nucleotide binding proteins [12, 15, 16]. There is clearly, therefore, a need for both a suitable ligand and binding procedure for use with mammalian purinoceptors. A number of studies have been reported investigating the binding of [^{35}S]-ADPβS to rat brain and liver [17–19]. However, the relationship between this binding and the P_{2y} purinoceptor is not clear as the characteristics and kinetics of [^{35}S]ADPβS binding were not reported.

We and others have recently reported that bovine

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‡ Abbreviations: [^{35}S]ADPβS, [^{35}S]adenosine 5′-*O*-(2-thiodiphosphate); PLC, phospholipase C; 2MeSATP, 2-methylthio ATP; β,γ-me ATP, β,γ-methylene ATP; BAE cells, bovine aortic endothelial cells; BAME cells, bovine adrenal medulla endothelial cells; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); BSS, balanced salt solution.

aortic endothelial cells contain co-existing P_{2y} and P_{2u} purinoceptors both of which were linked to PLC activation [20–23]. Furthermore, we have shown that ADP and ADPβS are neither agonists nor antagonists at P_{2u} purinoceptors [21, 22, 24], that is the effects of ATP are mediated via both receptor types while ADP acts only at the P_{2y} purinoceptor. Because of the attributes of [^{35}S]ADPβS, listed above, this radioligand was chosen as a suitable ligand for use in the development of a binding assay to study the P_2 purinoceptors present on endothelial cells. Binding assays were performed using standard filtration techniques and a novel procedure designed to study binding under equilibrium conditions. Binding at equilibrium was thought to be important as it was assumed that the affinity of ADPβS would be in the micromolar range (the stimulation of PLC activity by ADPβS has an EC_{50} of 0.85 μM) and a substantial amount of binding would therefore be lost with conventional assays.

The experiments described in this paper were carried out to attempt a thorough characterization of the binding of [^{35}S]ADPβS to mammalian cells and to study the pharmacology of the P_2 purinoceptors present on cultured endothelial cells.

MATERIALS AND METHODS

Cell culture. BAE cells were prepared from fresh aortae, obtained from a local abattoir, by digestion with 2–5 mg/mL collagenase [25] and cultured in 175 cm^2 tissue culture flasks (Nunc) in Minimal Essential Medium D-valine, with 10% foetal calf serum supplemented with 25 IU/mL penicillin, 25 μg/mL streptomycin, 10 mg/mL gentamycin, 250 μg/mL fungizone and 27 mg/mL glutamine and maintained at 37° in a water saturated 95% air, 5% CO_2 atmosphere. For experiments with cells in suspension, cultures were grown in 175 cm^2 flasks, while for equilibrium binding experiments, cells were grown in 24 well multiplates. At confluence, cells formed a cobblestone monolayer which was positive for Factor VIII immunofluorescence.

BAME cells were dissociated from intact glands by collagenase and protease treatment and subsequently purified and collected as described previously [26]. Non-adherant cells, mainly chromaffin cells, were decanted and the adherant cells cultured in the above medium until confluent. The cells when confluent exhibited the same morphological characteristics and growth patterns as described by Banerjee *et al.* [27].

Equilibrium binding procedure. Separation of free from bound radioligand for low affinity ligands would lead to a loss of a large percentage of binding sites due to disruption of the equilibrium between the two [28]. In an attempt to overcome these problems and to measure low affinity binding sites for [^{35}S]-ADPβS we devised a simple equilibrium binding procedure whereby bound and free radioligand can be measured separately. This involves sampling two compartments, one containing only free ligand and the other containing both bound and free. Bound ligand can then be computed as the difference between these two.

Cells grown in 24 well multiplates (approximately 50–75 μg cell protein per well), were washed twice with BSS without magnesium, comprising (in mM): NaCl, 135; KCl, 5.4; $NaHCO_3$, 16.2; D-glucose, 5.5; HEPES, 30; Na_2PO_4, 1.0; $CaCl_2$, 1.8; buffered to pH 7.4 with NaOH and gassed with 95% O_2, 5% CO_2. Incubations were initiated by the addition of [^{35}S]ADPβS (10^{-10}–10^{-9} M) and various concentrations of other ligands in a total volume of 400 μL. Incubations were for up to 4 hr at 4° after which a 200 μL aliquot of the supernatant was removed to a scintillation vial, to provide an estimate of free radioligand concentration. The reaction was then terminated by the addition of 250 μL of 0.2 M NaOH to the cells and the remaining incubate. The wells were then frozen and thawed and the contents scraped and removed to a scintillation vial thus providing an estimate of bound plus remaining free radioligand. Subtraction of free from bound plus free gives a direct measure of bound radioligand. At equilibrium there was approximately 26.5% bound radioactivity which was found in two affinity sites.

Filtration binding procedure. Confluent cells were grown in 175 cm^2 flasks and washed with fresh medium, which was then replaced with 10 mL of medium with 20% foetal calf serum. Cells were harvested by scraping the monolayer and centrifuged at 200 *g* for 10 min at room temperature. The medium was removed and the cells were resuspended by trituration in 10 mL of BSS without magnesium (see above composition) and spun again at 200 *g* for 10 min. The cells were finally resuspended in 2 mL BSS without magnesium and the protein concentration was adjusted to between 50 and 75 μg per 50 μL aliquot. Aliquots were incubated in a final volume of 150 μL containing [^{35}S]ADPβS (10^{-10}–10^{-9} M) and ligands as described. Incubations were carried out for up to 4 hr at 4° in BSS without Mg^{2+}. Experiments were terminated by rapid filtration and samples were collected on Whatman GF-B glass fibre filters. Samples were immediately washed three times with 5 mL of ice cold BSS minus Mg^{2+}. Filters were allowed to air dry and counted in scintillation vials.

Determination of non-specific binding and protein concentration. Non-specific binding for both procedures was determined by the amount of [^{35}S]-ADPβS bound in the presence of 10^{-4} or 10^{-3} M non-radioactive ADPβS. This ranged between 5 and 12% of total binding. Protein concentration was determined by the method of Lowry *et al.* [29].

Materials. [^{35}S]ADPβS (1000–1200 Ci/mmol) was purchased from NEN DuPont (UK) Ltd (Herts, U.K.). 2MeSATP was from Research Biochemicals (Herts, U.K.). Other chemicals were from Sigma (U.K.) or from Fisons (U.K.). Cell culture materials were from GIBCO.

RESULTS

Association and dissociation

Cells were incubated with [^{35}S]ADPβS (5×10^{-10} M) for up to 5 hr at 4°; apparent equilibrium was reached between 3 and 4 hr. Dissociation was initiated by the addition of 10^{-4} M unlabelled ADPβS in a 10-fold excess. Figure 1(A) shows association and dissociation curves for

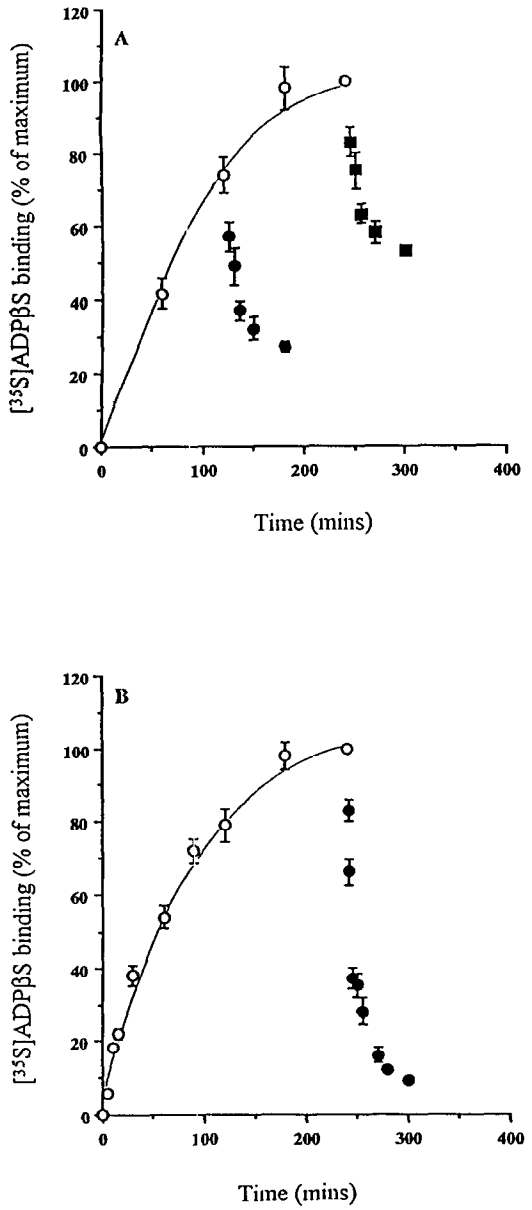


Fig. 1. Association (open circles) and dissociation (closed circles) time courses for binding of [^{35}S]ADPβS to cultured bovine aortic endothelial cells using either the equilibrium method (A) or the filtration method (B). Cells were incubated with [^{35}S]ADPβS (5×10^{-10} M) for the times indicated to determine association. To determine dissociation cells were incubated for 2 or 4 hr (A) or 4 hr (B) and then an excess of unlabelled ADPβS (10^{-4} M final concentration) was added to initiate dissociation. Data are mean ± SEM of three experiments carried out in duplicate.

experiments carried out using the equilibrium procedure. After incubation for 4 hr at 4° to reach equilibrium, only approximately 50% of the specific bound radioactivity was found to be displaceable after 1 hr. For example, in one triplicate experiment specific binding was 55225 ± 3224 dpm at 4 hr, after the addition of 10^{-4} M unlabelled ADPβS, 30593 ± 1244 dpm were remaining after a further hour of incubation. If the addition of unlabelled ADPβS took place after 2 hr of incubation with radioligand (giving approximately 75% of maximum bound), then 1 hr after its addition approximately 25% of the label specifically bound at 2 hr remained (8138 ± 992 dpm). The absolute amount of radioactivity lost after 60 min dissociation initiated at either 2 or 4 hr was 24414 ± 2054 dpm and 24633 ± 1992 dpm, respectively (N = 3 from one triplicate experiment). When the incubation was terminated by filtration (Fig. 1(B)), there was a rapid decline in radiolabel, one hour after addition of 10^{-4} M unlabelled ADPβS, nearly all the specific radioactivity had been lost.

Saturation analysis of [^{35}S]*ADPβS binding*

Saturation analysis was carried out with a constant concentration of [^{35}S]ADPβS (10^{-10} M) and increasing concentrations of unlabelled ADPβS (10^{-10}–10^{-4} M), followed by isotope dilution analysis to compute the amount of ligand bound (pmol/mg protein) at each ligand concentration.

Figure 2(A) and 2(B) show the untransformed data for equilibrium and filtration experiments respectively. In Fig. 2(C), the data are expressed as a competition curve, enabling a direct comparison of the effect of the two procedures on the position and shape of the binding curves. There was a steepening of the curve from a Hill coefficient of 0.58 ± 0.06 (equilibrium) to one of 0.75 ± 0.03 (filtration) and leftward shift of the IC_{50} from $2.3 \pm 1.2 \times 10^{-7}$ M to $1.7 \pm 0.03 \times 10^{-8}$ M, respectively (N = 5 in each case).

In Fig 3(A) and 3(B) isotope dilution analysis is shown represented as Scatchard plots. Iterative curve fitting of these data using the EBDA–ligand curve fitting program [30] was used to generate values of K_D and B_{max}. Data were best fit to two site models in each case. Equilibrium binding analysis produced a high affinity site with K_D $3.32 \pm 0.14 \times 10^{-8}$ M and B_{max} 32.4 ± 14.5 pmol/mg protein and a low affinity site with K_D $4.3 \pm 2.0 \times 10^{-6}$ M and B_{max} 2155 ± 789 pmol/mg protein. Filtration analysis gave a high affinity site with K_D $1.58 \pm 0.25 \times 10^{-8}$ M and B_{max} 14.25 ± 8.9 pmol/mg protein and a low affinity site of K_D $2.39 \pm 0.5 \times 10^{-6}$ M and B_{max} 103.3 ± 33.7 pmol/mg protein. The values of K_D for both sites was not significantly different between experimental protocols ($P < 0.05$), neither was the B_{max} value of the high affinity site. There was, however, a loss of the majority of the low affinity site when filtration was used to terminate the incubation compared with the equilibrium technique.

These experiments accurately quantified the low affinity binding site using the equilibrium protocol. However, due to the lack of difference between the K_D values with the two procedures the remaining experiments were performed with the less time consuming filtration procedure.

Competition curves for [^{35}S]*ADPβS binding*

Analogues of ATP, ADP and UTP inhibited [^{35}S]ADPβS binding in a competitive manner (Fig. 4). The overall rank order of agonist potency was as follows: ADPβS > ATPγS >> ATP > β,γ-

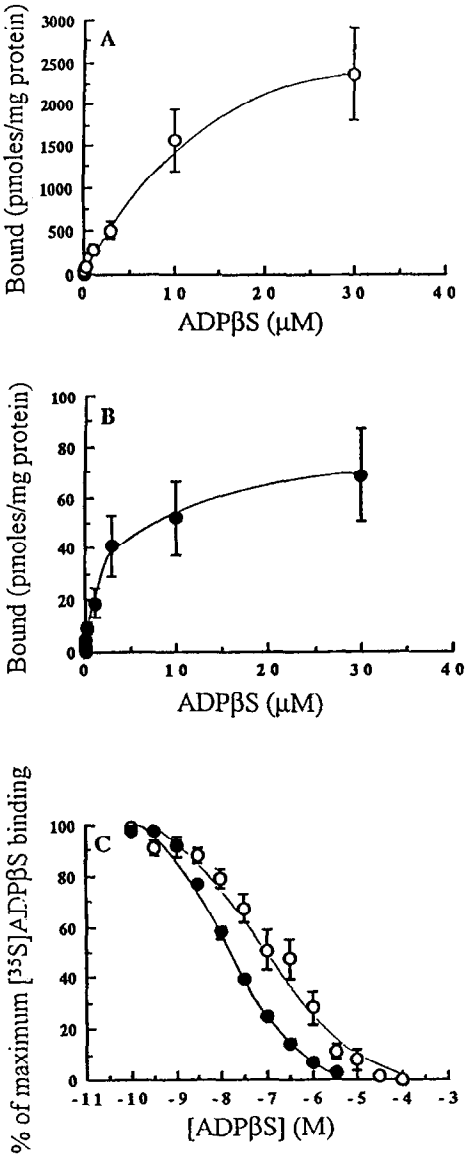


Fig. 2. Saturation analysis of [^{35}S]ADPβS binding. Bovine aortic endothelial cells were incubated with radioligand (10^{-10} M) and increasing concentrations of unlabelled ADPβS for 4 hr at 4° and the reactions were terminated by either the equilibrium (A) or filtration (B) methods and expressed as a saturation curve after isotope dilution analysis. In C, the data from A and B are expressed as a competition curve for the equilibrium (open circles) and filtration (closed circles) experiments, normalized to a % of maximum binding. Data are mean ± SEM of four or five triplicate experiments.

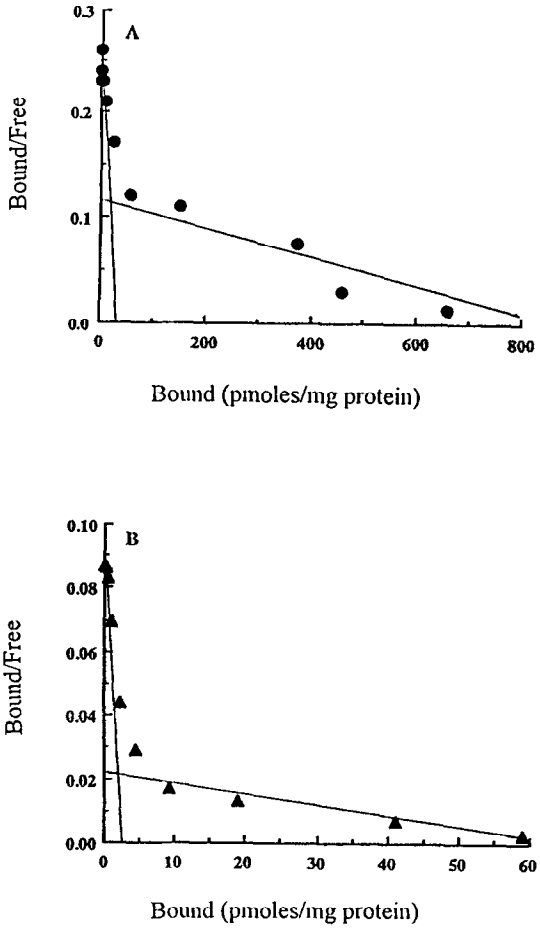


Fig. 3. Scatchard plots of saturation binding data obtained by isotope dilution analysis from Fig. 2. (A) equilibrium, (B) filtration method. Data are representative of four or five triplicate experiments.

meATP > 2MeSATP > UTP. Values for IC_{50} and Hill coefficient are shown in Table 1. With the exception of ADPβS, the Hill slopes were not significantly different from unity. However, the potency order was not that expected from the presence of a P_{2y} purinoceptor as 2MeSATP was less potent than β,γ-meATP and ATP in competing for [^{35}S]ADPβS binding sites and β,γ-meATP was able to displace the majority of the binding. The residual binding with maximal displacement by β,γ-meATP varied between 5 and 20% of maximal binding.

In studies not reported here we have shown that β,γ-meATP, at a concentration of 10^{-4} M, did not interfere with concentration–response curves to ADPβS for stimulation of total [^{3}H]inositol (poly)phosphates in [^{3}H]inositol labelled BAE cells indicating that β,γ-meATP acts as neither an agonist nor an antagonist at the P_{2y} purinoceptor (Wilkinson GF and Boarder MR, unpublished observations). We therefore undertook binding studies in the presence of β,γ-meATP (10^{-4} M) in order to attempt to reduce the large amount of non-specific binding observed above (Fig. 5(A)). Specific binding was reduced by between 80 and 85% and the IC_{50} for competition for [^{35}S]ADPβS (10^{-10} M) binding was shifted from $1.7 \pm 0.3 \times 10^{-8}$ M to $5.76 \pm 0.7 \times 10^{-6}$ M in the presence of β,γ-meATP (N = four to five triplicate experiments). Iterative curve fitting produced a best fit to a one site model with an apparent K_D of $5.35 \pm 1.3 \times 10^{-6}$ M (N = 4).

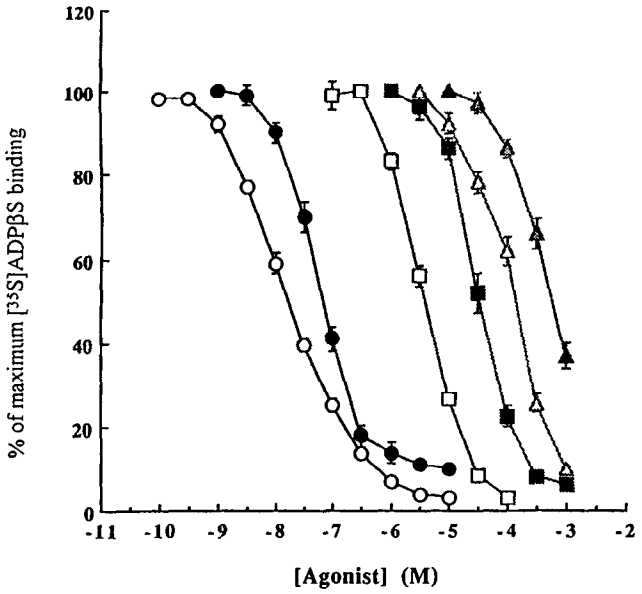


Fig. 4. Concentration dependent competition for [^{35}S]ADPβS binding by a number of purine nucleotides and UTP. Bovine aortic endothelial cells were incubated with radioligand (10^{-10} M) for 4 hr at 4° with the indicated concentrations of ADPβS (○), ATPγS (●), ATP (□), β,γ-meATP (■), 2MeSATP (△) and UTP (▲). Experiments were terminated by filtration. Data are expressed as a percentage of the maximum specific bound and are the mean ± SEM of three triplicate experiments.

Table 1. Potency of a number of P_2 purinoceptor agonists for competing with [^{35}S]ADPβS binding

Agonist	pIC_{50}	Hn	N
ADPβS	7.77 ± 0.34	0.75 ± 0.03	6
ATPγS	7.24 ± 0.76	1.03 ± 0.15	5
ATP	5.47 ± 0.45	0.98 ± 0.08	5
β,γ-meATP	4.22 ± 0.34	0.96 ± 0.07	5
2MeSATP	3.99 ± 0.25	1.14 ± 0.17	4
UTP	3.62 ± 0.26	1.13 ± 0.14	3

BAE cells in suspension were incubated for 4 hr at 4° in BSS minus magnesium with [^{35}S]ADPβS (10^{-10} M) and increasing concentrations of the above agonists. After this time the incubation was terminated by filtration and rapid washing with three 5 mL vol of ice cold BSS minus Mg^{2+}. Data are mean ± SEM from three to six separate experiments each carried out in triplicate. Data were analysed using the graph pad curve fitting program to determine pIC_{50} (i.e. $-\log_{10} IC_{50}$).

Competition curves were also carried out in the presence of β,γ-meATP (Fig 5(B)) and gave the following rank order of agonist potency; ATPγS = ADPβS >> 2MeSATP > UTP (N = three triplicate experiments). UTP failed to produce complete displacement of radiolabel at a concentration of 10^{-3} M.

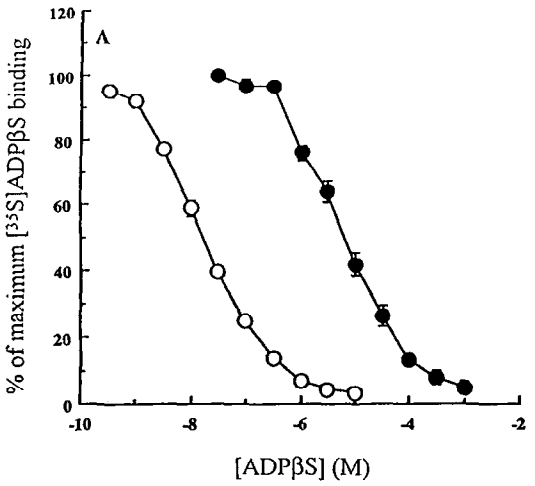
Binding of [^{35}S]ADPβS to BAME cells

We have previously shown that BAME cells do not possess PLC linked P_{2y} purinoceptors, but do express P_{2u} purinoceptors, and that ADPβS does not act as agonist on these cells [26, 31]. However, as can be seen in Fig. 6, [^{35}S]ADPβS (10^{-10} M) is bound to an apparently homogenous population of binding sites on BAME cells in suspension with an IC_{50} of $6.3 \pm 0.5 \times 10^{-7}$ M and Hill coefficient of 0.92 ± 0.03 (N = three duplicate experiments).

DISCUSSION

The aims of this study were two-fold. First, we wished to study the characteristics of the P_{2y} purinoceptors which have been functionally described on cultured BAE cells using a radioligand binding approach. Secondly, we wished to study the characteristics of the binding of [^{35}S]ADPβS, the radiolabel used in these studies, to determine its suitability for binding in a mammalian cell system, as the only thorough study previously carried out was in avian erythrocytes [12].

It has been shown that agonists for the P_{2y} purinoceptor on cultured endothelial cells have EC_{50} values for the stimulation of inositol (poly)phosphate production in the micromolar range, indeed ADPβS has an EC_{50} in BAE cells of 0.85 ± 0.4 μM [21]. It would seem likely, therefore, that the binding affinity of these agonists to their receptors would also produce K_D values in the micromolar range. This would, therefore, place restraints on the choice of procedure for separation of bound from free radioligand if an unacceptable amount of bound radiolabel was not to be lost during the termination of an experiment. It has been shown by Yamamura *et al.* [28] that for a radiolabel with K_D of 10^{-6} M,



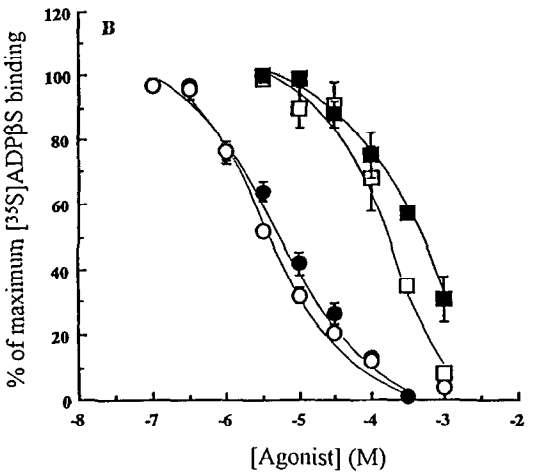


Fig. 5. Concentration-dependent competition of [^{35}S]-ADPβS binding in the presence of β,γ-methylene ATP. Bovine aortic endothelial cells were incubated for 4 hr at 4° with [^{35}S]ADPβS (10^{-10} M) and β,γ-meATP (10^{-4} M). (A) The effect of co-incubation with β,γ-meATP on the curve to ADPβS, open circles in the presence and closed circles in the absence of β,γ-meATP. (B) The effect of co-incubation with β,γ-meATP on competition curves for ATPγS (○), ADPβS (●), 2MeSATP (□) and UTP (■). Experiments were terminated by filtration. Data are the mean ± SEM of three triplicate experiments.

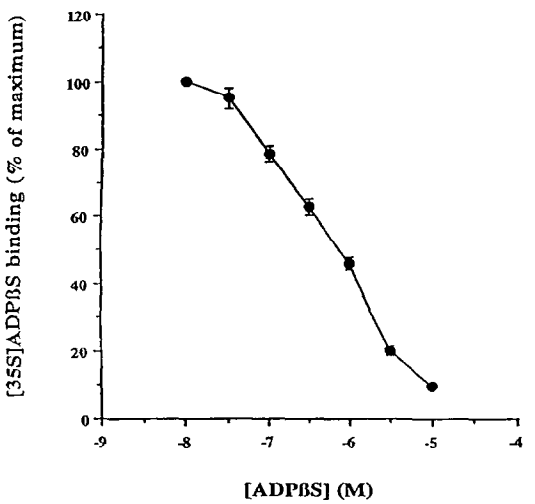


Fig. 6. Concentration dependent competition for [^{35}S]-ADPβS binding to bovine adrenal medulla endothelial cells. Cells in suspension were incubated for 4 hr at 4° with radioligand (10^{-10} M) and increasing concentrations of unlabelled ADPβS. Experiments were terminated by filtration. Data are the mean ± SEM of three triplicate experiments.

and an assumed association rate constant of 10^6/M/sec that 10% of bound label would be lost in 0.01 sec, compared to 17 min for a ligand with a K_D of 10^{-10} M. We therefore developed a simple equilibrium binding technique for use with intact cells in monolayer culture to try and overcome these perceived problems and compared results from this technique with those obtained with standard filtration procedures.

Interpretation of agonist binding studies is intrinsically complex, especially when applied to intact cells, due in part to the presence of uncontrolled guanine nucleotide concentrations affecting the affinity of seven transmembrane receptors and also due to the possibility of desensitization and internalization of receptors in the presence of agonists. An indication of the complexity of the binding is apparent in this report with respect to the dissociation of bound radioactivity as seen with the equilibrium method (see Fig. 1(A)). When a large excess of unlabelled ADPβS was added to initiate dissociation, only a part of the bound radioactivity was displaced into the free compartment. The absolute amount displaced remained the same between 2 and 4 hr of prior incubation. During which time the total specific bound and the non-specific binding both increased. This non-displaceable binding may represent internalized ligand. This seems unlikely when results with equilibrium and filtration procedures are compared. Such results have been seen in other studies of agonist binding to whole cells. When dissociation studies were carried out with the filtration procedure, nearly all the bound radioligand was displaceable, contrary to the results obtained with equilibrium studies. This increase in displaceable ligand may be due to an alteration in the equilibrium between bound and free radioligand, i.e. when the incubation was terminated by rapid washing the previously non-displaceable bound radioligand moved into a pool of radioligand which was able to participate in the equilibrium between bound and free, and hence to be displaced by washing.

Both techniques allowed the definition of two sites with different affinities. Only the low affinity site was affected by the procedure used, in line with the points raised above, with over 95% of the B_{max} lost when the incubation was terminated by filtration. The presence of two affinity sites has been described

by other workers for the binding of [^{35}S]ADPβS and [^{35}S]ADPγS in mammalian whole cell and membrane preparations, e.g. heart sarcollema membranes [8], human neutrophils [32], rat brain synaptic vesicles [19] and rat hepatocytes [17]. In all of these cases the K_D and B_{max} values were in the same range described here.

The low affinity binding site had an apparent K_D of between 2.4 and 4.3×10^{-6} M, close to the EC_{50} for stimulation of inositol (poly)phosphate production in these cells. However, the concentration of binding sites was unrealistically large. The receptor may be present but masked by a large amount of non-receptor binding sites. Endothelial cells possess other extracellular non-receptor sites for ATP and ADP such as ectonucleotidases [33] and kinases [34]. The termination of the incubation with filtration reduced the number of these sites, but at 100 pmol/mg protein the number was still unrealistically large for a receptor. High B_{max} values for P_2 purinoceptor binding sites have been described in other mammalian cells, ranging from 51 pmol/mg protein in rat hepatocytes for [^{35}S]ADPβS binding [17], to between 812 and 2995 pmol/mg protein in rat sarcolemma membranes [8]. It would therefore seem likely that the functional sites in these systems were also being masked by substantial non-receptor binding.

Another attempt to reduce non-receptor binding was the use of β,γ-meATP in experiments. This P_{2x} purinoceptor agonist has no activity at either the P_{2y} or P_{2u} purinoceptors found on BAE cells, but was found to be more potent than 2MeSATP in competing for [^{35}S]ADPβS binding. The effects of this agonist on the binding of ligands to PLC linked P_2 purinoceptors in other mammalian systems has not been reported so it is difficult to compare its effects with those described here. Binding of [^{35}S]ADPβS in the presence of β,γ-meATP produced a shift in the affinity estimate for ADPβS to a one site model with a K_D in the low micromolar range, consistent with that expected from concentration–response curves for these agonists, with EC_{50} values around 1 μM. However, the rank order of agonist potency, although changed with respect to ADPβS and ATPγS, still did not correspond to that expected of a P_{2y} purinoceptor due to the lack of ability of 2MeSATP to displace binding in the presence of β,γ-meATP. 2MeSATP has been shown to be the most potent agonist for the stimulation of inositol (poly)phosphates [21] but was less potent at displacing of [^{35}S]ADPβS binding than either ADPβS or β,γ-meATP. Such low potency in binding assays has been reported in other studies. For instance, in rat hepatocytes, 2MeSATP was 25 times more potent than ATP at stimulating glycogen phosphorylase activity, but displaced [^{35}S]ADPγS binding with much lower potency than ATP [35] and in rat synaptic membranes 2MeSATP has been shown to be the least potent agonist in displacing [^{35}S]ADPβS binding [18]. These results could be taken as indicating that in these systems 2MeSATP was a low affinity agonist with a high receptor reserve, in which case the potency of 2MeSATP in such binding assays would be expected to be to the right of the EC_{50} for activation of inositol phosphate metabolism.

However, the observation that, in the presence of 10^{-4} M β,γ-meATP, UTP displaces [^{35}S]ADPβS binding with a potency similar to that of 2MeSATP is difficult to reconcile with binding mainly to the P_{2y} purinoceptor. An alternative interpretation is that binding in the presence of β,γ-meATP is the residue of the same binding sites in its absence. This is unlikely because the displacement curve with unlabelled ADPβS was right shifted in the presence of β,γ-meATP and the order of potency of ADPβS and ATPγS was reversed. These points suggest that the nature of the binding in the presence of β,γ-meATP was fundamentally different from that occurring in its absence.

Overall these studies indicate that [^{35}S]ADPβS cannot be used for P_{2y} purinoceptor radioligand binding assays without effective removal of residue binding to other sites. This is seen in our results with BAE cells and with binding to BAME cells which have no P_{2y} purinoceptors [31] and presumably reflects results seen in many published studies. However, our results also show that it is possible to remove most of this other binding and that under these circumstances [^{35}S]ADPβS may be a useful probe for P_{2y} purinoceptor.

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