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Evidence that most high-affinity ATP binding sites on aortic endothelial cells and membranes do not correspond to P_2 receptors

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Abstract

It has recently been demonstrated that two types of ATP receptors, the P_{2Y} and P_{2U} receptors, are coexpressed on bovine aortic endothelial cells. The aim of the present study was to characterize directly P_{2Y} and P_{2U} subtypes on intact bovine aortic endothelial cells and on membranes prepared from these cells using adenosine 5'-0-(3-thio[^{35}S]triphosphate) ([^{35}S]ATPγS), [α-^{32}P]ATP and [α-^{32}P]UTP as radioligands. [^{35}S]ATPγS binding to bovine aortic endothelial cell membranes was saturable and apparently involved a single class of high-affinity binding sites (K_d: 14 ± 11 nM; B_{max} 1.6 ± 0.7 pmol/mg protein; mean ± S.D.). A similar class of high-affinity binding sites was identified with [α-^{32}P]ATP (K_d: 14 ± 9 nM; B_{max}: 1.7 ± 1.1 pmol/mg protein; mean ± S.D.). Competition experiments showed that only one third of these sites bound 2-methylthio-ATP (2-MeSATP) with high affinity (K_i: 21 ± 5 and 14 ± 10 nM, mean ± S.D., for [^{35}S]ATPγS and [α-^{32}P]ATP, respectively) and might therefore represent the P_{2Y} receptors. UTP did not compete with [^{35}S]ATPγS or [α-^{32}P]ATP for binding at the remaining sites, indicating that they are not the P_{2U} receptors. No high-affinity UTP binding sites could be detected using [α-^{32}P]UTP. [^{35}S]ATPγS binding to intact bovine aortic endothelial cells was competed by ATPγS (K_d: 1.0 ± 0.5 μM; mean ± S.D.), but not by 2-MeSATP and UTP, indicating that these binding sites are neither the P_{2Y} nor the P_{2U} receptors.

Keywords: ATP; Purinergic; Nucleotide; Purinoceptor; Endothelial cell

1. Introduction

Extracellular ATP plays an important role in the regulation of prostacyclin and nitric oxide release from vascular endothelial cells (Boeynaems and Pearson, 1990). Two types of ATP receptors, both coupled to phospholipase C activation, are involved in these actions: the P_{2Y} and P_{2U} receptors (Motte et al., 1993a; Wilkinson et al., 1993). In the absence of selective antagonists, the distinction between these two subtypes of P_2 receptors is based on the rank order of potency of various agonists. The P_{2Y} receptor exhibits a selectivity for adenine nucleotides: the order of potency is 2-methylthio-ATP (2-MeSATP) > ATP = ADP, whereas UTP and other pyrimidine nucleotides are inactive (Burnstock and Kennedy, 1985; O'Connor, 1992; Fredholm et al., 1994). By contrast with the P_{2Y} receptor, the P_{2U} subtype does not exhibit an absolute specificity for purines and is characterized by the following rank order of potency: ATP = UTP > ITP > CTP, ADP and UDP being weak agonists as compared to the respective nucleotide triphosphate (O'Connor, 1992; Fredholm et al., 1994). The relative contribution of P_{2Y} and P_{2U} receptors to the action of ATP is variable from one type of endothelial cells to the other. In microvascular endothelial cells from bovine adrenal medulla (Allsup and Boarder, 1990; Purkiss et al., 1993), rabbit myocardium (Mannix et al., 1993) and rat brain (Frelin et al., 1993), the inositol phosphate response to ATP seems to be mediated exclusively by P_{2U} receptors. On the contrary, the bovine aortic endothelial cell line AG4762 expresses only P_{2Y} receptors (Allsup and Boarder, 1990). The two types of receptors coexist on bovine aortic endothelial cells (Motte et al., 1993a; Wilkinson et al., 1993). The arguments supporting the involvement of both P_{2U} and P_{2Y} receptors in the inositol phosphate response of bovine aortic endothelial cells to ATP are the following:

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the effects of 2-MeSATP and UTP were additive whereas the effects of ATP and either UTP or 2-MeSATP were not; ATP desensitized the responses to both UTP and 2-MeSATP, whereas there was only a minimal cross-desensitization between 2-MeSATP and UTP; pertussis toxin inhibited the action of UTP but had no effect on the response to 2-MeSATP.

The aim of the present study was to characterize directly P_{2Y} and P_{2U} subtypes on intact bovine aortic endothelial cells and on cell membranes prepared from these cells, using adenosine 5′-0-(3-thio[^{35}S]triphosphate) ([^{35}S]-ATPγS), [α-^{32}P]ATP and [α-^{32}P]UTP as radioligands.

2. Materials and methods

2.1. Cell culture and membrane preparation

Bovine aortic endothelial cells were obtained by collagenase digestion of the aorta excised from a freshly slaughtered cow and cultured as previously described (Pirotton et al., 1991).

When the culture formed a confluent monolayer, the cells were washed with ice-cold buffer (NaCl: 150 mM; KCl: 3 mM; Na_2HPO_4: 6 mM; KH_2PO_4: 1 mM), scraped and then disrupted in ice-cold lysis buffer (Tris-HCl: 50 mM, pH 7.5; $MgCl_2$: 2 mM; EDTA: 0.3 mM; EGTA: 1 mM; leupeptin: 5 mg l^{-1}; phenylmethanesulphonylfluoride: 50 mg l^{-1}) using a Teflon/glass homogenizer. All procedures for the membrane preparation were performed at 4°C. The membrane suspension was centrifuged at 24000 × g for 30 min. The supernatant was discarded and the pellet was resuspended, homogenized again and centrifuged (24000 × g, 30 min). The pellet was suspended in Tris-HCl 50 mM (pH 7.5) and stored in aliquots at −80°C until used. Aliquots were used within 2 weeks following the preparation and were thawed just before each experiment. Results were reproducible between aliquots of the same preparation, suggesting that the suspended pellet stored at −80°C was stable. The protein concentration of the final suspension was determined by the method of Lowry with bovine serum albumin as a standard.

2.2. Binding to cell membranes

Binding assays of [^{35}S]ATPγS, [α-^{32}P]ATP and [α-^{32}P]UTP were carried out in Tris-HCl (50 mM, pH 7.5), EDTA 1 mM, in a final volume of 0.5 ml, containing 25–50 μg of protein and 0.5 nM of radioligand. In general, the assays were conducted at 30°C for 5 min. Incubations were stopped by the addition of 4 ml of ice-cold Tris-HCl (50 mM, pH 7.5) and rapid filtration through Whatman GF/B filters under reduced pressure. The filters were then washed three times with 2 ml of the same ice-cold Tris-HCl buffer. Radioactivity was quantified by liquid scintillation counting, after an overnight incubation of the filters in liquid scintillation mixture. In all competition experiments, data are expressed as a percentage of maximal specific binding of [^{35}S]ATPγS, [α-^{32}P]ATP or [α-^{32}P]UTP and non-specific binding was defined with an excess of the unlabeled homologous ligand (1 μM ATPγS, 1 μM ATP and 100 μM UTP, respectively). The total specific binding of [^{35}S]ATPγS and [α-^{32}P]ATP represented 2.5 ± 1.1 and 3.4 ± 0.8% (mean ± S.D.) of the total amount of radioligand, whereas the amount of radioligand trapped by the filter was less than 0.2%. However, variations in the buffer composition affected the radioligand binding to the filters. Addition of $MgCl_2$ (5 mM) and $AlCl_3$ (10 μM) resulted in an increase in filter-bound radioactivity (to 8 and 14%, respectively, of the total radioactivity added), whereas the addition of NaF (20 mM) had no effect.

2.3. Measurement of nucleotide degradation

To characterize the degradation of [^{35}S]ATPγS, [α-^{32}P]ATP and [α-^{32}P]UTP, reactions were stopped as described above except that after the filtration, the incubation medium and the washes were analysed by ion-pair reversed-phase HPLC on a μBondapack C_{18} column as described by Cascalheira and Sebastiano (1992). [^{35}S] and [^{32}P] radioactivity in the fractions (15 s) was quantified by liquid scintillation counting. The peaks were identified by the co-elution of unlabeled standard nucleotides.

2.4. Binding to intact bovine aortic endothelial cells

Binding assays to intact bovine aortic endothelial cells were performed as described by Kim and Rabin (1994), with some modifications. Briefly, cells were washed three times with Ca^{2+}- and Mg^{2+}-free buffer (124 mM NaCl, 5 mM KCl, 8 mM D-glucose, 1.25 mM KH_2PO_4, 1 mM EDTA and 25 mM Na-Hepes, pH 7.4) and then scraped in the same buffer. The cells were collected by centrifugation at 1500 × g for 2 min, resuspended and preincubated for 20 min at room temperature in the same buffer. The binding assays of [^{35}S]ATPγS were carried out in the same buffer supplemented with 1 mM EGTA, in a final volume of 0.5 ml, containing 0.5 to 1 × 10^6 cells and 2 nM of radioligand. After a 5 min incubation period at room temperature, a 0.4 ml aliquot of the suspension was layered onto a Whatman GF/B filter and washed three times with 2 ml of ice-cold Ca^{2+}- and Mg^{2+}-free buffer.

2.5. Cell incubation and measurement of inositol phosphate accumulation

Bovine aortic endothelial cells were cultured as previously described (Pirotton et al., 1991). Subconfluent cells were labeled for 24 h in inositol-free Dulbecco's modified Eagle's medium (DMEM) containing fetal calf serum (5%, v/v), penicillin at 100 U ml^{-1}, streptomycin at 100 μg

ml^{-1} and amphotericin B at 2.5 $\mu g\ ml^{-1}$, supplemented with myo-D-[^{3}H]inositol at 10 $\mu Ci\ ml^{-1}$. The cells were washed twice with DMEM and incubated in this medium for 30 min before the addition of the agonist. The incubation was stopped 15 s later. At that time, most of the inositol trisphosphate ($InsP_3$) fraction represents Ins(1,4,5)P_3 (Pirotton et al., 1991). The inositol phosphates were extracted as described previously (Pirotton et al., 1991). Briefly, the medium was rapidly replaced by 1 ml of $HClO_4$ (3%, w/v). The dishes were rinsed with 1 ml of $HClO_4$ (1%) and the lysate was neutralized with KOH (0.765 M) and Hepes (0.375 M). The inositol phosphates were separated on Dowex AG1X8 columns (0.8 ml wet bed volume) as previously described.

2.6. Materials

The radioactive products [^{35}S]ATPγS (> 1000 Ci/mmol), [α-^{32}P]ATP (3000 Ci/mmol), [α-^{32}P]UTP (3000 Ci/mmol) and myo-D-[2-^{3}H]inositol (10–20 Ci/mmol) were from Amersham (Ghent, Belgium). Proteinase inhibitors (leupeptin, phenylmethanesulphonylfluoride), collagenase type IA and all nucleotides were obtained from Sigma Chemical (St. Louis, MO, USA), with the exception of 2-MeSATP which was purchased from RBI (Natick, MA, USA). All culture media and reagents were purchased from GIBCO (Grand Island, NY, USA). Dowex AG1X8 (formate form) was from Bio-Rad Laboratories (Richmond, CA, USA). The μBondapack C_{18} column was from Waters-Millipore (Milford, USA). Filters were from Whatman International (Maidstone, UK). The liquid scintillation mixture (Instagel II plus) was from Packard Instrument (Meriden, USA).

2.7. Data analysis

All experiments were carried out in triplicate. Non-linear least squares curve-fitting was used to analyse competition curves and to determine IC_{50}. Alternative one-site and two-site binding models were tested in all cases. The determination of the dissociation constant K_i of a competing ligand was based on the equation of Cheng and Prussof $IC_{50} = K_i(1 + [L]/K_d)$, where $[L]$ and K_d are the free radioligand concentration and its dissociation constant, respectively. In the case of heterologous displacement, $K_i = IC_{50}/(1 + [L]/K_d)$. K_i can be approximated by IC_{50} because $[L]$ is low as compared to K_d in our experiments. In the case of homologous displacements, the equation simplifies to $K_d = IC_{50} - [L]$. Again, K_d can be approximated by IC_{50} because $[L]$ is much lower than the IC_{50}. The concentration of receptor sites or B_{max} (maximal specific binding) was estimated by using the following equation: $B_{max} = (b/l)IC_{50}$, where b is the specifically bound radioligand in the absence of competitor, l is the amount of radioligand contained in the sample and IC_{50} the concentration of homologous unlabeled ligand needed to inhibit binding by 50% (Swillens, 1992).

3. Results

3.1. Binding of [^{35}S]ATPγS and [α-^{32}P]ATP to bovine aortic endothelial cell membranes

The binding of [^{35}S]ATPγS increased rapidly during the first few minutes and a plateau value was reached within 3 min as shown in Fig. 1. The addition of 1 μM unlabeled ATPγS resulted in rapid (within 3 min) dissociation of the radioligand (data not shown). Fig. 2 shows that the binding increased linearly with the membrane protein concentration and Fig. 3 shows that [^{35}S]ATPγS binding, measured after 5 min, was saturable and apparently involved a single class of high-affinity binding sites (K_d: 22.6 nM; B_{max} 1.9 pmol/mg protein). The binding of [^{35}S]ATPγS was inhibited in a concentration-dependent manner by unlabeled ATPγS and ATP (Fig. 4A). Results of non-linear regression analysis were compatible with the existence of a single class of high-affinity binding sites and showed that [^{35}S]ATPγS binding was inhibited by ATPγS (K_d: 14 ± 11 nM; B_{max}: 1.6 ± 0.7 pmol/mg; mean ± S.D. of 7 experiments) as well as by ATP (K_i: 16 ± 12 nM; mean ± S.D. of 7 experiments). As shown in Fig. 4B, closely similar results were obtained using [α-^{32}P]ATP as radioligand. Binding occurred to a single class of high-affinity binding sites and was inhibited by ATP (K_d: 14 ± 9 nM; B_{max}: 1.7 ± 1 pmol/mg; mean ± S.D. of 5 experiments) as well as by ATPγS (K_i: 15 ± 5 nM; mean ± S.D. of 3 experiments). HPLC analysis showed that the presence of EDTA was crucial to slow down the degradation of [α-^{32}P]ATP: 55% of the added radioligand was recovered intact after a 3 min incubation in the

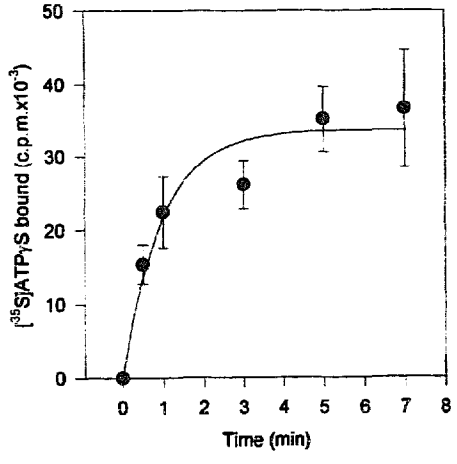

Fig. 1. Time course of [^{35}S]ATPγS binding. Bovine aortic endothelial cell membranes were incubated for the indicated times at 30°C in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA with 0.5 nM [^{35}S]ATPγS. Results represent the mean of triplicate determinations in 1 representative experiment out of 4.

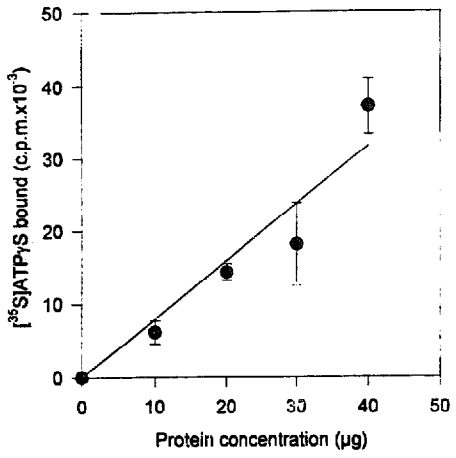

Fig. 2. Linearity of [^{35}S]ATPγS binding to bovine aortic endothelial cell membranes with protein concentration. Increasing concentrations of bovine aortic endothelial cell membranes were incubated for 5 min at 30°C in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA with 0.5 nM [^{35}S]ATPγS. Results are the mean of triplicate determinations in 1 representative experiment.

presence of EDTA, whereas the degradation of the radioligand was virtually complete within 1 min in the absence of EDTA (Fig. 5). EDTA was also required to reduce the degradation of [^{35}S]ATPγS since in the absence of EDTA, only 44% of the total amount of [^{35}S]ATPγS was recovered after a 5 min incubation as compared to 80% in the presence of EDTA. Competition experiments with a wide range of nucleotides were performed in an attempt to establish a relationship between [^{35}S]ATPγS/[α-^{32}P]ATP binding to the membranes and the functional evidence that P_{2Y} and P_{2U} receptors coexist on bovine aortic endothelial cells. Fig. 6 shows that the binding of both [^{35}S]ATPγS

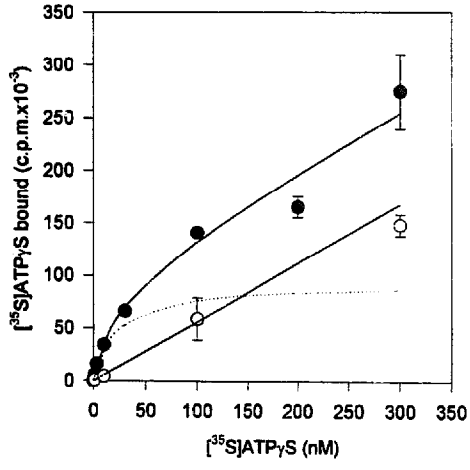

Fig. 3. Saturability of [^{35}S]ATPγS binding to bovine aortic endothelial cell membranes. Bovine aortic endothelial cell membranes were incubated for 5 min at 30°C in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA with increasing concentration of [^{35}S]ATPγS (0.3–300 nM). Non-specific binding was defined as the residual binding measured in the presence of a large excess of unlabeled ATPγS (300 μM). Results represent the mean of triplicate determinations in 1 representative experiment. Total binding (●); non-specific binding (○); calculated specific binding (dotted line).

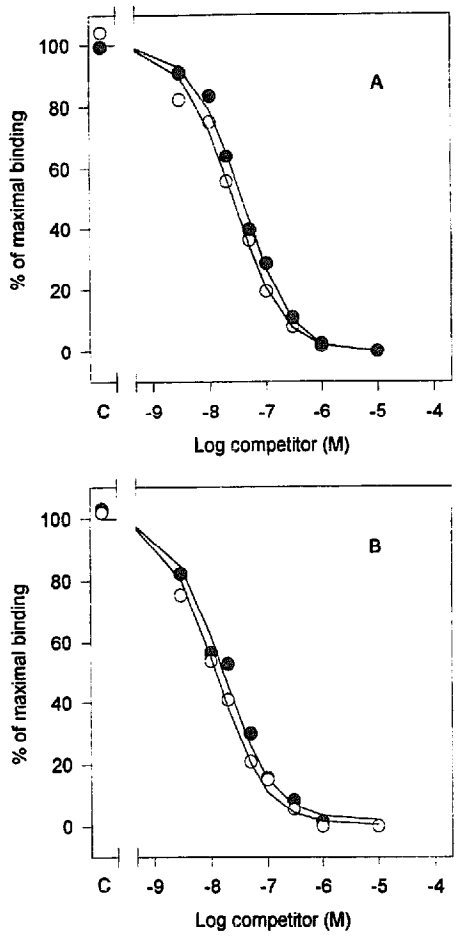

Fig. 4. Concentration-dependent competition of [α-^{32}P]ATP and [^{35}S]ATPγS binding to bovine aortic endothelial cell membranes by ATP and ATPγS. Bovine aortic endothelial cell membranes were incubated for 5 min at 30°C in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA with 0.5 nM [^{35}S]ATPγS (A) or [α-^{32}P]ATP (B) and various concentrations of ATP (●) or ATPγS (○). Results are the mean of triplicate determinations in 1 representative experiment out of 7 for each radioligand.

and [α-^{32}P]ATP was inhibited in a biphasic manner by 2-MeSATP. A two-site model best fitted these data and identified a high-affinity binding site for 2-MeSATP representing 37% (mean of 3 experiments; range 26–47%) of the [α-^{32}P]ATP total specific binding (K_i: 14 ± 10 nM; mean ± S.D. of 3 experiments) and 38% (range 27–55%) of the total [^{35}S]ATPγS specific binding (K_i: 21 ± 5 nM; mean ± S.D. of 3 experiments). In the presence of adenosine-5′-0-2 thiodiphosphate ($ADP_\beta S$), previously used to characterize the avian P_{2Y} receptor (Cooper et al., 1989), non-linear regression analysis again showed that a two-site model best fitted the data for the two radioligands (Fig. 6, Table 1). The orders of potency of other adenine nucleotide analogues to compete with both radioligands were similar (table 1) and were as follows: ATP = ATPγS > 2′- and 3′-0-(4-benzoylbenzoyl) adenosine 5′-triphosphate (BZATP) = 2-chloroadenosine 5′-triphosphate (2-ClATP) ≥ ADP = 5′-adenylyl imidodiphosphate (AMP-PNP) > adenosine 5′-($\alpha\beta$-methylene)-triphosphate

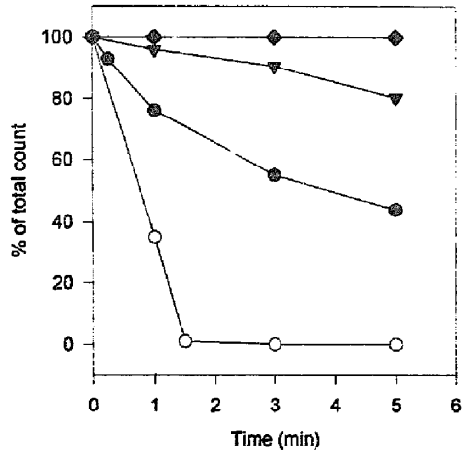

Fig. 5. Time course of [^{35}S]ATPγS, [α-^{32}P]ATP and [α-^{32}P]UTP degradation during an incubation with bovine aortic endothelial cell membranes. Bovine aortic endothelial cell membranes were incubated at 30°C for up to 5 min with [^{35}S]ATPγS (in the presence of EDTA) (▼), [α-^{32}P]UTP (in the presence of EDTA) (◆) or [α-^{32}P]ATP with (●) or without (○) EDTA (1 mM). Following filtration of the membrane suspension, an aliquot of the medium was injected on a μBondapack C_{18} column, which was eluted as described elsewhere (Cascalheira and Sebastiano, 1992). Fractions (15 s) were collected and the ^{35}S or ^{32}P radioactivity was measured by liquid scintillation counting; the peaks were identified by the co-elution of unlabeled nucleotides. Results represent the percentage of added nucleotide recovered unchanged (mean values of duplicate determinations in 1 representative experiment).

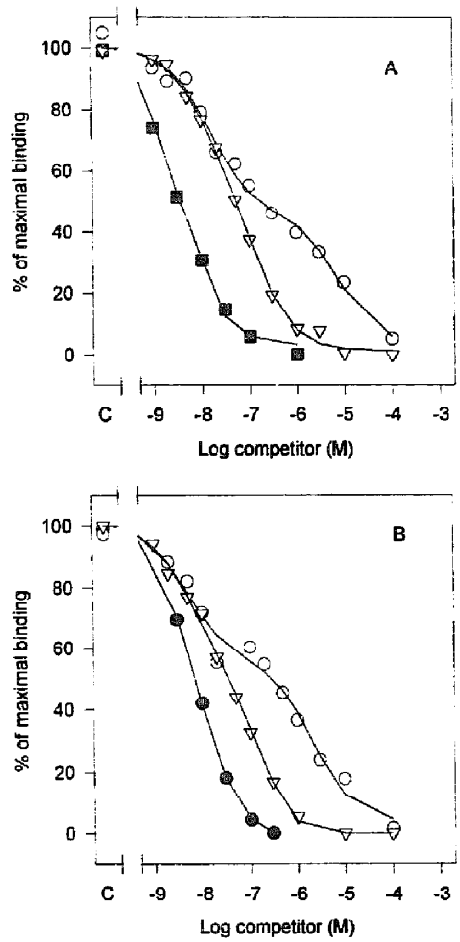

Fig. 6. Concentration-dependent competition of [^{35}S]ATPγS and [α-^{32}P]ATP binding to bovine aortic endothelial cell membranes by ATP, 2-MeSATP and ADPβS. Bovine aortic endothelial cell membranes were incubated in the presence of 0.5 nM [^{35}S]ATPγS (A) or 0.5 nM [α-^{32}P]ATP (B) and various concentrations of ATPγS (■), ATP (●), 2-MeSATP (○) and ADPβS (▽) (mean values of triplicate determinations in 1 representative experiment out of 3).

(αβMeATP) ≥ adenosine 5′-(βγ-methylene)-triphosphate (βγMeATP) ≫ AMP. The diadenine nucleotides AP_4A and AP_3A were also tested: they had a low potency, the K_i values being 0.1 and 1 μM, respectively (data not shown). The non-adenine nucleotides GTPγS, CTP and, notably, UTP had a very low potency, with $K_i > 3$ μM (Fig. 7). Finally, various xanthine antagonists of adenosine receptors, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX),

Table 1
Comparison of the ability of various ATP and ADP analogs to inhibit [^{35}S]ATPγS and [α-^{32}P]ATP binding to bovine aortic endothelial cell membranes

Competitors	K_i [mean (range)] nM	
	[^{35}S]ATPγS	[α-^{32}P]ATP
ATP	16 (5–38)	14 (3–25)
ATPγS	14 (3–32)	15 (12–19)
2-MeSATP	21 (15–24) 13 600 (7 240–20 000)	14 (5–24) 3 300 (2 200–4 600)
ADPβS	19 (11–26) 224 (119–329)	6 (4–7) 322 (155–480)
BZATP	49 (45–52)	37 (32–43)
2-ClATP	42 (35–50)	44 (26–63)
ADP	98 (151–45)	41 (11–70)
AMPPNP	103 (101–105)	36 (24–49)
αβMeATP	473 (151–795)	121 (120–123)
βγMeATP	1096 (792–1 400)	355 (102–608)
AMP	> 10 μM	> 10 μM

K_i values (mean, range) were obtained from competition binding curves, using non-linear least-squares curve fitting of at least 2 independent experiments for each competitor. In the case of 2-MeSATP and ADPβS, data were significantly better fitted using a two-site model than a single-site one: the K_i values for the 2 components are given.

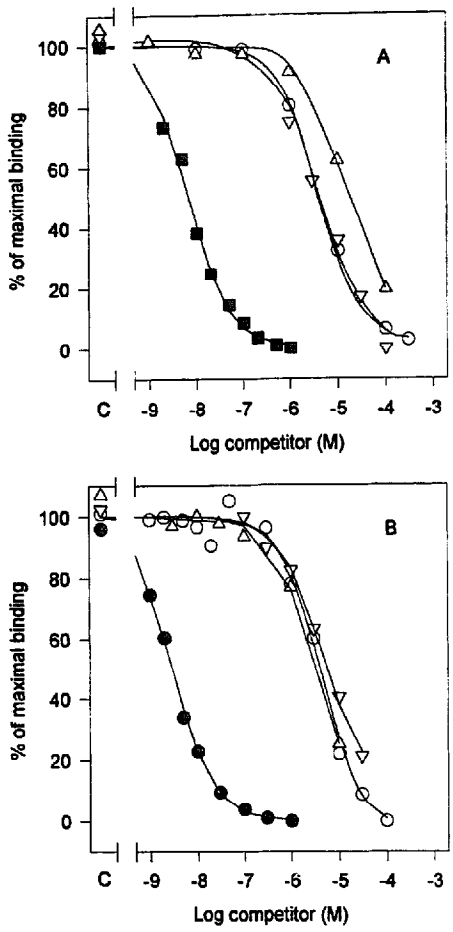

Fig. 7. Competition of [^{35}S]ATPγS and [α-^{32}P]ATP binding to bovine aortic endothelial cell membranes by UTP, CTP and GTPγS. Bovine aortic endothelial cell membranes were incubated for 5 min in the presence of 0.5 nM [^{35}S]ATPγS (A) or 0.5 nM [α-^{32}P]ATP (B) and various concentration of ATPγS (■) ATP (●), UTP (○), CTP (△) or GTPγS (▽) (mean values of triplicate determinations in 1 representative experiment out of 7).

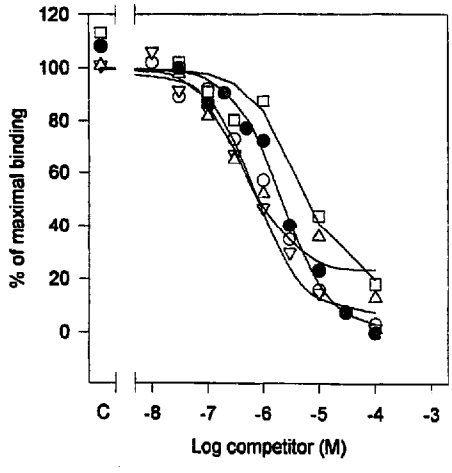

Fig. 8. Competition of [α-^{32}P]UTP binding to bovine aortic endothelial cell membranes by various nucleotides. Bovine aortic endothelial cell membranes were incubated, as described under Materials and methods, for 5 min with 0.5 nM [α-^{32}P]UTP in the presence of various concentrations of UTP (●), ATP (○), UDP (▽), CTP (□) or ITP (△) (mean values of triplicate determinations in 1 representative experiment out of 8).

xanthine amine congener (XAC), 8-(*p*-sulfophenyl)-theophylline (8PST) and 8-cyclopentyl-theophylline (CPT) did not compete with the binding of [α-^{32}P]ATP, even at a concentration of 10 μM (data not shown).

3.2. Binding of [α-^{32}P]UTP to bovine aortic endothelial cell membranes and correlation with inositol phosphate accumulation

Binding of [α-^{32}P]UTP increased rapidly during the first few min and reached a plateau value within 3 min (not shown), in the same way as [α-^{32}P]ATP and [^{35}S]ATPγS. However, binding assays with [α-^{32}P]UTP failed to detect a high-affinity binding site (Fig. 8) (K_d: 1.45 ± 0.5 μM; B_{max}: 26 ± 9.5 pmol/mg; mean ± S.D. of 10 experiments). This could not be attributed to a rapid

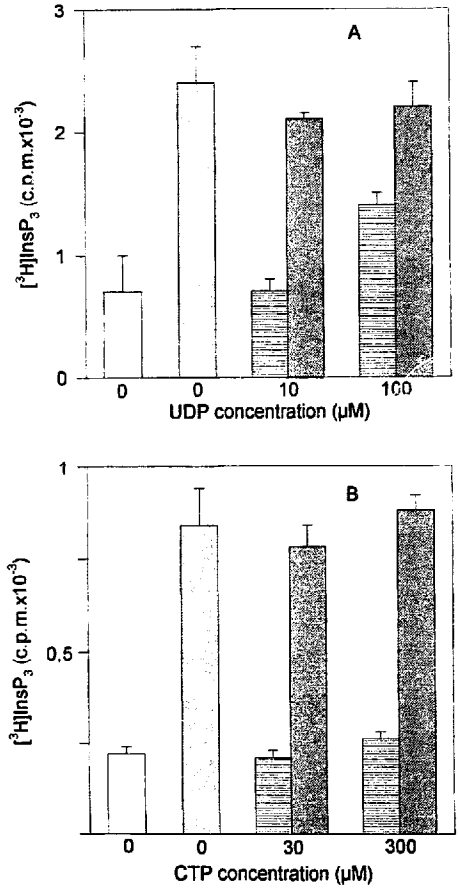

Fig. 9. Effect of UDP and CTP on the accumulation of inositol trisphosphate ($InsP_3$) in bovine aortic endothelial cells and its stimulation by UTP. A: Bovine aortic endothelial cells, prelabeled with [^{3}H]inositol, were challenged for 15 s with UTP either alone or in combination with UDP. B: Prelabeled bovine aortic endothelial cells were challenged either with UTP alone or simultaneously with UTP and CTP. The $InsP_3$ fraction was isolated as described in Materials and methods. Data from 1 experiment out of 2 are expressed as mean ± S.D. of triplicate dishes. Bars indicate the following conditions: control (open bar), 50 μM UTP (hatched), UDP alone (horizontal shading), UDP+50 μM UTP (cross-hatched). Panel B: control (open bar) 30 μM UTP (hatched), CTP alone (horizontal shading), CTP+30 μM UTP (cross-hatched).

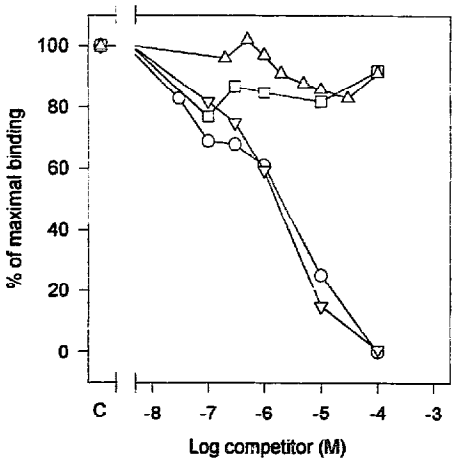

Fig. 10. Competition of [^{35}S]ATPγS binding to intact bovine aortic endothelial cells by various nucleotides. Intact bovine aortic endothelial cells were incubated, as described in Materials and methods, in the presence of 2 nM [^{35}S]ATPγS and various concentration of ATPγS (○), ATP (▽), 2-MeSATP (△) and UTP (□) (mean values of triplicate determinations in 1 representative experiment out of 2).

metabolism of the radioligand since HPLC analysis showed that [α-^{32}P]UTP was not degraded throughtout the incubation period (Fig. 5). Pre-treatment of the membranes with increasing concentrations of Triton (0.01 to 1%) did not unmask cryptic high-affinity sites. Moreover, the rank order of potency observed in competition studies did not correspond to that observed for the activation of phospholipase C. In particular, UDP and CTP were able to compete with [α-^{32}P]UTP binding. However, UDP barely induced inositol phosphate accumulation and CTP was totally inactive; furthermore neither compound was a competitive antagonist of UTP (Fig. 9).

3.3. Binding of [^{35}S]ATPγS to intact bovine aortic endothelial cells

Fig. 10 shows that [^{35}S]ATPγS binding to intact bovine aortic endothelial cells, in the presence of 1 mM EDTA and 1 mM EGTA, was inhibited in a concentration-dependent manner by unlabeled ATPγS (K_d: 1.0 ± 0.5 μM; mean ± S.D. of 5 experiments) and ATP. However, the number of binding sites per cell was high ($1.3 \times 10^6 \pm 0.2 \times 10^6$; mean ± S.D. of 5 experiments) and neither 2-MeSATP nor UTP (at concentrations up to 100 μM) did compete with the binding of [^{35}S]ATPγS.

4. Discussion

Some features of the ATP binding sites that we have detected on bovine aortic endothelial cell membranes are not inconsistent with the hypothesis that they might represent P_2 receptors. The high-affinity site, with K_d close to 10 nM, is comparable to that observed in the most convincing radioligand binding studies of P_2 receptors performed so far. In turkey erythrocyte membranes, where Cooper et al. (1989) labeled putative P_{2Y} receptor sites using [^{35}S]ADPβS, the K_i for ATP was 101 nM. More recently, Simon et al. (1995) measured the binding of [^{35}S]dATPαS to membranes of transfected COS-7 cells expressing the chick P_{2Y1} receptor: in that model the K_i for ATP was 48 nM. The number of ATP binding sites on bovine aortic endothelial cell membranes was also reasonable: about 1.7 pmol/mg protein, a value comparable to the 3 pmol/mg figure found in turkey erythrocytes (Cooper et al., 1989), and much less than the surprisingly high number of [^{35}S]ATPαS sites found in hepatocyte membranes (from 24 to 71 pmol/mg, depending on the species) (Keppens and De Wulf, 1986; Keppens et al., 1989, 1990). We and others have shown that the P_2 receptors on endothelial cells, both P_{2Y} and P_{2U}, are activated by a form of ATP uncomplexed to divalent cations (presumably ATP^{4-}) and not by $ATPMg^{2-}$, which predominates in biological fluids and constitutes the substrate of kinases and ATPases (Motte et al., 1993b; Lustig et al., 1992). Therefore, our binding assay was carried out in a Mg^{2+}- and Ca^{2+}-free medium containing EDTA in order to reduce the binding of ATP to non-receptor sites. Furthermore the inclusion of EDTA was required to slow down radioligand degradation, but it was unable to prevent it completely. This is consistent with the report that a significant activity of the endothelial ecto-ATPase is maintained in the absence of divalent cations (Pearson et al., 1980). The metabolic instability of the ligand,in particular [α-^{32}P]ATP, precludes accurate determination of the K_d and leads to some underestimation of the affinity of the competitors. In our opinion, this does not affect our conclusion that both radioligands [^{35}S]ATPγS and [α-^{32}P]ATP are able to identify a class of high-affinity binding sites. Since the results obtained with [^{35}S]ATPγS and [α-^{35}P]ATP were closely similar, we can exclude the possibility that we actually measured the binding of labeled ADP or AMP formed from [α-^{32}P]ATP. We can also exclude a binding of [^{35}S]ATPγS to thiophosphate binding sites as observed in human neutrophils (Yu et al., 1991).

The competition of [^{35}S]ATPγS and [α-^{32}P]ATP binding to bovine aortic endothelial cell membranes by 2-MeSATP was clearly biphasic. In the case of ADPβS, the existence of an intermediary plateau in the competition curve was less conspicuous, but nevertheless a two-site model allowed a significantly better fitting of the data than a single-site model. It is tempting to speculate that the fraction of the binding sites with a high affinity for 2-MeSATP and ADPβS (±37% of the total) represents P_{2Y} receptors.It is worth noting that the biphasic displacement by 2-MeSATP revealed the proportion of sites labeled by the radioligand in the absence of competitor but not the actual proportion of high- and low-affinity binding site densities (Swillens et al., 1995). For these high-affinity

sites, the K_i of 2-MeSATP was not lower than that of ATP itself, whereas functional studies (endothelium-dependent relaxation or $[Ca^{2+}]_i$ rise) indicate that 2-MeSATP is more potent than ATP at endothelial P_{2Y} receptors (Martin et al., 1985; Needham et al., 1987; Carter et al., 1988). However, relative affinities of agonists do not necessarily correlate when binding and functional assays are compared (Hulme and Birdsall, 1992). Moreover, 2-MeSATP and ATP were equipotent competitors (K_i: 69 and 48 nM, respectively) of [^{35}S]dATPαS binding to membranes of transfected COS-7 cells expressing the chick brain P_{2Y} receptor (Simon et al., 1995). Another problem is that GTPγS behaved as a weak competitor of [^{35}S]ATPγS or [α-^{32}P]ATP binding to bovine aortic endothelial cell membranes, in a similar way as UTP or CTP, whereas in turkey erythrocytes it was a potent non-competitive inhibitor of [^{35}S]ADPβS binding, presumably as a result of uncoupling between P_{2Y} receptors and a G-protein. This might suggest that the ATP binding sites detected on bovine aortic endothelial cell membranes are not G-protein-coupled receptors. However, the lack of a significant GTPγS effect could result from the removal of divalent cations by EDTA: it is indeed well established that Mg^{2+} is involved at various steps in the interaction between receptors and G-proteins (Hulme and Buckley, 1992). We attempted to mimic the effect of GTPγS with AlF^{4-} or to use GTPγS in the presence of Mg^{2+}. However, this resulted in an artefactual binding of cation-complexed ATP to the filters (see Materials and methods).

Whether or not the fraction of sites to which 2-MeSATP binds with high affinity represents P_{2Y} receptors, it is clear that the remaining sites are not P_{2U} receptors, since the K_i of UTP was > 3 μM, that is more than 200-fold greater than the K_i of ATP. The affinity of adenine dinucleotides for these sites was also very low, suggesting that they are unrelated to the P_{2D} receptors, detected inter alia in synaptic terminals (Pintor et al., 1993). The lack of competition by various xanthines also indicates that these sites are distinct from the putative methylxanthine-sensitive receptors which mediate the stimulatory effect of ATP on adenylyl cyclase in bovine aortic endothelial cells (Côte et al., 1993) as well as in other cell types (Sato et al., 1992; Tada et al., 1992).

We also failed to directly detect P_{2U} binding sites using [α-^{32}P]UTP as radioligand. This could not be attributed to a rapid metabolisation of the radioligand since HPLC analysis showed that it was not degraded throughout the incubation period. The binding observed was of low affinity and the rank order of potency of various nucleotides for competition at these sites did not correspond to that observed for phospholipase C activation. In particular, UDP was equipotent to UTP as a binding competitor, but it had little effect on inositol phosphates accumulation: it neither fully mimicked nor antagonized the stimulatory effect of UTP. We must therefore conclude that the low-affinity binding sites detected with [α-^{32}P]UTP are not a low-affinity state of the P_{2U} receptors. It might be that the P_{2U} receptors are trapped on the inner face of vesicles formed during membrane preparation, precluding binding of the radioligand. However, our attempts to unmask cryptic UTP binding sites using detergents were unsuccessful.

The risk of labeling non-receptor sites with radiolabeled nucleotides should be less with intact cells than with membranes. Therefore, we performed radioligand binding studies with intact bovine aortic endothelial cells, using a method similar to that recently described by Kim and Rabin (1994). We detected binding sites of reasonable affinity, but there was no competition by either 2-MeSATP or UTP. This is reminiscent of the recent work of Wilkinson and Boarder (1995) showing that the affinity of both 2-MeSATP and UTP was very low for [^{35}S]ADPβS binding sites on intact bovine aortic endothelial cells. This suggests that these binding sites represent non-receptor sites. With PC12 cells, Kim and Rabin (1994) also failed to detect a competition of [^{35}S]ATPαS by nucleotides other than ATP, ATPαS and ATPγS and concluded to the existence of atypical P_2 receptors on these cells. Our failure to detect P_{2U} binding sites on intact bovine aortic endothelial cells is also reminiscent of the work of Yu et al. (1991). In human neutrophils, there was a good correlation between the ability of various nucleotides to compete with the binding of [^{35}S]ATPγS and to raise $[Ca^{2+}]_i$, except for UTP, which was a potent agonist but a very weak competitor. Recently, Van Rhee et al. (1993) reported that UTP was equipotent to ATP as a competitor of [^{35}S]ADPβS binding to intact hepatocytes. However, these data must be interpreted with caution since in most models ADPβS is not a P_{2U} agonist.

In conclusion, whereas functional studies have established the presence of both P_{2Y} and P_{2U} receptors on bovine aortic endothelial cells, radioligand binding studies performed with intact bovine aortic endothelial cells and bovine aortic endothelial cell membranes failed to detect P_{2U} receptors. It might be that a fraction of the binding sites labeled in membranes are P_{2Y} receptors, but this conclusion is only based on the high affinity of these sites for 2-MeSATP and ADPβS. Binding studies relying on the use of radiolabeled nucleotides must be interpreted with extreme caution as underscored by the recent paper of Filtz et al. (1994). These authors failed to detect an increase in [^{35}S]ADPβS binding to 1321N1 astrocytoma cell membranes following transfection with a construction encoding the turkey P_{2Y1} receptor. Furthermore, they acknowledged that a more extensive analysis of [^{35}S]ADPβS binding to turkey erythrocytes membranes, using a wider range of competing ligands, led them to question the validity of their previous conclusion that these binding sites represent genuine P_{2Y} receptors. In a similar way, our study illustrates the complexity of radioligand binding studies of P_2 receptors and suggests that no convincing data will be obtained before labeled high-affinity antagonists become available.

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