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# Differential Characterization of Binding Sites for Adenine and Uridine Nucleotides in Membranes from Rat Lung as Possible Tools for Studying P2 Receptors in Lung

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**Abstract.** Nucleotide receptors (P2 receptors) are involved in stimulating $Cl^-$ secretion in airway epithelia. These receptors may play a key role in development of new therapeutic strategies in the treatment of cystic fibrosis. However, the diversity of nucleotide binding sites in lung tissue has not yet been clarified. Here we studied the characteristics of various nucleotide binding sites in rat lung membranes by equilibrium binding analysis of several P2 receptor specific ligands. Displacement studies revealed a recognition site for adenosine 5′-*O*-(1-thiotriphosphate) ([$^{35}$S]ATPαS; $K_d$ 243 nM). From this site the ligand is readily displaced by adenosine 5′-*O*-(2-thiodiphosphate) (ADPβS), a typical agonist for $P2Y_1$ receptors and also by α,β-methylene adenosine 5′-triphosphate (α,β-MeATP), a typical agonist for P2X receptors. [$^3$H]α,β-MeATP labelled specific binding sites ($K_d$ 56 nM) in rat lung membranes. Analysis of binding of [$^3$H]UTP to lung membranes revealed a high-affinity binding site ($K_d$ 44 nM). Membrane-bound [$^3$H]UTP was not displaced even by high concentrations of ATP, indicating no common binding site for UTP and ATP. Furthermore, specific binding of P-1,P-4-di(adenosine 5′)tetraphosphate ([$^3$H]$Ap_4A$; $K_d$ 91 nM) was found in lung membranes. Thus, we demonstrate at least four distinct types of nucleotide binding sites in lung membranes: Two have characteristics comparable to P2X and $P2Y_1$ receptors, while two further sites still have to be identified, one binding $Ap_4A$ and the other binding UTP very specifically. BIOCHEM PHARMACOL **55**;5:687–695, 1998. © 1998 Elsevier Science Inc.

**KEY WORDS.** adenosine 5′-triphosphate; P-1,P-4-di(adenosine 5′)tetraphosphate; lung membranes; nucleotide receptors; P2 receptors; uridine 5′-triphosphate

Nucleotides such as ATP† and UTP play an important role in signal transduction in a variety of cells and tissues. Although these nucleotides only occur at extremely low concentrations in the extracellular milieu, they can exert profound effects when binding to specific receptor proteins in the plasma membrane. Receptors that bind ATP and/or ADP are classified as P2 receptors (for nomenclature see [1]). These receptors are involved in numerous biological processes such as regulation of phospholipase C [2], $Ca^{2+}$ mobilization [3], chloride secretion in human airway epithelial cells [4], and neuromodulation in a variety of cells and tissues [5, 6]. Investigating physiological responses to nucleotides in neural cells, we have found that UTP induces $[Ca^{2+}]_i$ oscillations in rat glioma cells [7]. In a neuronal cell line, the ligands UTP and ATP cause different desensitization mechanisms on $P2Y_2$ receptors [8] (formerly called $P_{2U}$ [12]).

According to the affinity of binding of ATP analogues, the P2 receptors were formerly subclassified into five pharmacologically distinct families ($P_{2T}$, $P_{2U}$, $P_{2X}$, $P_{2Y}$, and $P_{2Z}$) [9, 10]. At least one of these subclasses ($P_{2U}$) is also activated by the pyrimidine nucleotide UTP [11–15]. Recently $P_{2U}$-purinoceptors, whose structure was elucidated by molecular cloning [12], were designated $P2Y_2$ receptors, indicating that these receptors are part of the receptor family coupled to a G-protein [16], in contrast to P2X receptors, which are ligand-gated ion channels. Until now, however, it has been difficult to determine whether the physiological effects of ATP or UTP are mediated by the same or different proteins. Cloning and expression of a $P2Y_2$ receptor provided evidence for a single receptor protein [17] activated by both ATP and UTP in an equipotent manner with the following rank order of affinities: UTP ≥ ATP > ATPγS > 2MeSATP > α,β-MeATP [10].

There is also evidence for the existence of additional

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† *Abbreviations:* ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); α,β-MeATP, α,β-methylene adenosine 5′-triphosphate; $Ap_4A$, P-1,P-4-di(adenosine 5′)tetraphosphate; ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); $B_{max}$, maximal number of binding sites; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane regulator; $K_d$, dissociation constant; $K_i$, inhibitory constant; 2-MeSATP, 2-methylthio adenosine 5′-triphosphate.

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receptors for UTP that do not fit into the usual classification scheme for P2 receptors [18–20]. A receptor responding primarily to uridine nucleotides was identified by molecular cloning [18]. Furthermore, notions concerning the occurrence of receptors specifically recognizing diadenosine polyphosphates ($P2Y_{AP4A}$, formerly called $P_{2D}$ [1]) are still controversial. $Ap_4A$ is a typical representative of the group of diadenosine polyphosphates that is found in a variety of tissues. $Ap_4A$ is proposed to be an endogenous regulator molecule of P2 receptors. In the nervous system, the existence of a distinct $P_{2D}$-purinergic receptor binding $Ap_4A$ was postulated [21]. However, data were provided demonstrating that $Ap_4A$ is also a $P2Y_2$ receptor agonist with a potency comparable to that of ATP and UTP [22].

Clinical studies show that UTP receptors seem to play an important role in the regulation of secretion of $Cl^-$ ions and surfactant in human lung [4, 23]. The finding that nucleotide receptors can regulate ion fluxes in epithelia from patients with cystic fibrosis (CF) was an important breakthrough in the rationale for the design of a novel drug therapy for the disease [24, 25]. In CF patients, $Cl^-$ secretion via $Cl^-$ permeability of the cystic fibrosis transmembrane conductance regulator (CFTR) is disturbed due to genetic defects in the CFTR gene. This impaired $Cl^-$ regulation leads to severe damage in the airway system and other exocrine organs such as pancreas and intestine. Both UTP and ATP are thought to stimulate $Cl^-$ channels via alternative mechanisms, not involving CFTR, in cystic fibrosis airways, thus circumventing the lack of CFTR-dependent $Cl^-$ secretion in the lung [25–27]. Therefore, UTP is considered a potent therapeutic agent for bringing relief to CF patients by the stimulation of ion transport processes that promote hydration of airway secretions.

The aim to rationalize the therapeutic aspects of nucleotide treatment prompted investigations of the regulation of cell functions controlled by extracellular UTP and other nucleotides, opening an important field of work related to the treatment of cystic fibrosis (mucoviszidosis) [4]. However, so far no biochemical or pharmacological investigations have been carried out to establish the identity and density of binding sites for various nucleotides in lung tissue. Knowledge concerning the nucleotide binding sites is of paramount importance for further physiological and clinical studies.

The major goal of our present work was to determine binding sites in lung membranes very specific for the ligand UTP and to provide a general characterization of additional binding sites for other nucleotides. Such data are necessary for evaluating nucleotide agents. Lung membranes seem to be a suitable starting material for the isolation of a UTP receptor and other nucleotide receptors. However, the density of various nucleotide binding sites in the lung membranes and the relative potency of ATP analogues and UTP are not yet known. By displacement studies, we could elucidate the existence of four different binding sites for purine or pyrimidine nucleotides in lung membranes of rat. The isolation of one of these sites which is very specific for UTP is under further investigation.

## MATERIALS AND METHODS

### *Materials*

ATP, ADPβS, α,β-MeATP, $Ap_4A$, and UTP were from Sigma, ATPαS from Calbiochem and 2-MeSATP from RBI. Radiolabelled [$^{35}$S]ATPαS (>1,000 Ci/mmol = 37 TBq/mmol) and [$^3$H]UTP (36.37 Ci/mmol = 1.3 TBq/mmol) were from DuPont NEN and [$^3$H]α,β-MeATP (26 Ci/mmol = 962 GBq/mmol) and [$^3$H]$Ap_4A$ (11.4 Ci/mmol = 422 GBq/mmol) were purchased from Amersham. All other chemicals were of the highest purity available.

### *Preparation of Lung Membranes*

Lungs dissected from 10–15 rats (*Rattus rattus*) were washed with sufficient amounts of ice-cold PBS (137 mM NaCl, 2.6 mM KCl, 8.1 mM $Na_2HPO_4$, and 1.4 mM $KH_2PO_4$, pH 7.4). Membranes, which were reported to contain mainly receptor-containing plasma membranes, were prepared according to the method of Mong [28], with some minor modifications described below. After removal of tracheae, lung tissue was homogenized in homogenization buffer containing 10 mM HEPES/NaOH, pH 7.4, 0.32 M sucrose, 0.5 mM benzamidine, 0.1 mM Pefabloc SC and 1 μg/mL leupeptin (6 mL buffer for 1 g of fresh weight) using an ultraturrax (45 sec in 3 intervals with half-maximal speed). The homogenate was centrifuged at 1,000 × *g* for 5 min to remove cellular debris. The pellet was resuspended, homogenized, and both supernatants collected. Membranes were pelleted by centrifugation (30,000 × *g*, 20 min) and purified by sucrose gradient centrifugation loading 25 mL homogenate onto 10 mL of 40% (w/v) sucrose in 25 mM HEPES, pH 7.4. After centrifugation at 100,000 × *g* for 90 min, membranes were collected from the interphase of the sucrose step gradient (11%/40%), diluted with an equal volume of 25 mM HEPES, pH 7.4, and pelleted by centrifugation at 150,000 × *g* for 30 min. The pellets were resuspended in homogenization buffer (0.1 mL per g of fresh weight) and stored at −80° until use. All steps were carried out at 4°. Protein content was determined according to Lowry *et al.* [29], with bovine serum albumin as standard.

### *Binding Experiments*

Binding of [$^{35}$S]- and [$^3$H]-labelled nucleotides was measured by incubation of lung membranes (25–50 μg of protein) in incubation medium (final volume 0.1 mL; 0.2 mL for the [$^3$H]UTP experiments) containing 25 mM HEPES, pH 7.4, 50 mM NaCl, and 5 mM KCl (binding buffer) for 35 min at 4° with increasing amounts of the radiolabelled nucleotide. Under these conditions, binding of radiolabelled nucleotides reached equilibrium and remained nearly constant for up to 60 min of incubation. For competition binding experiments, binding of a constant

amount of the labelled nucleotide was determined in the presence of increasing concentrations of various unlabelled nucleotides. After incubation, membranes were collected by centrifugation at 25,000 × *g* for 12 min. To remove unbound radioligand, pelleted membranes were quickly washed twice (within 10–15 sec) with 150 μL of binding buffer. Further washes did not affect the amount of ligand bound to membranes. Finally, membranes were resuspended in 100 μL of 1.5% (w/v) SDS. Membrane-bound radioactivity was measured using a liquid scintillation β-counter (Canberra Packard 1600 TR) with 1 mL of scintillator fluid (ULTIMA-Gold, Packard). Nonspecific binding was determined in the presence of 100 μM of the same unlabelled nucleotide.

### *Data Analysis*

The binding data of saturation and displacement binding were analyzed by nonlinear regression using the RADLIG program (RADLIG program, Biosoft Corp.) with models using one or two binding sites. Unless stated otherwise, experiments were performed with triplicate determinations.

## RESULTS

### *Saturation Binding of [$^3$H]UTP, [$^3$H]Ap$_4$A, and [$^3$H]α,β-MeATP*

To determine the density of nucleotide binding sites in membranes of rat lung, we incubated the membranes with radioactive-labelled purine and pyrimidine nucleotide agonists at concentrations ranging from 0.1 to 200 nM. The binding data of these saturation studies were analyzed by nonlinear regression using models with one or two binding sites. This analysis yielded a single binding site for each of the nucleotides under investigation. The $K_d$ values were 43.7 ± 9.1 nM ($N$ = 4) for UTP, 56.5 ± 10.0 nM ($N$ = 4) for α,β-MeATP, and 91.2 ± 8.8 nM ($N$ = 4) for Ap$_4$A. Maximal binding capacities, $B_{max}$, were 1.6 ± 0.9 pmol/mg protein for UTP, 5.7 ± 1.0 pmol/mg protein for α,β-MeATP, and 6.9 ± 1.6 pmol/mg protein for Ap$_4$A, respectively.

A comparison of the binding capacities which were achieved with the different nucleotide ligands showed that specific binding of α,β-MeATP and Ap$_4$A to lung membranes was considerably higher than that of UTP (Fig. 1A). UTP had a relatively high nonspecific binding of approximately 60% in lung membranes, whereas in the case of α,β-MeATP and Ap$_4$A, nonspecific binding in the presence of 100 μM unlabelled nucleotide was less than 30% of the total binding (data not shown).

### *Density of Nucleotide Binding Sites*

At ligand concentrations of 10 nM, lung membranes showed a very high binding activity for ATPαS, defined as specific binding at a given ligand concentration, with 5.33 ± 1.70 pmol/mg protein and somewhat lower specific binding activities for α,β-MeATP and Ap$_4$A, with comparable values of 1.34 ± 0.26 pmol/mg protein and 0.94 ± 0.13 pmol/mg protein, respectively. The high density of ATPαS binding sites might indicate binding to several types of receptors and binding proteins. We still have to clarify to what extent non-receptor proteins, such as enzymes or transporters, are also partly responsible for this binding.

On the other hand, we detected only a low binding activity for UTP with 0.14 ± 0.05 pmol/mg protein (Fig. 1B). We found the same relation for the binding activities of 1:10:7 for the specific binding sites for UTP, α,β-MeATP, and Ap$_4$A respectively, over a large range of ligand concentrations. This relation was fairly constant even up to 200 nM of nucleotide concentration, where saturation of binding was reached (data not shown).

### *Characterization of the [$^{35}$S]ATPαS Binding Sites*

The inhibition of binding of 1 nM [$^{35}$S]ATPαS to lung membranes was analyzed by testing a number of agonists which are active at P2 receptors. Data for displacement of bound [$^{35}$S]ATPαS by unlabelled ATPαS could only be fitted to a model assuming the presence of a single binding site with a $K_i$ value of 243.7 ± 87.9 nM and a $B_{max}$ value of 164.7 ± 27.6 pmol/mg protein. Figure 2A shows that [$^{35}$S]ATPαS was readily displaced both by α,β-MeATP, a specific ligand of P2X receptors, and by ADPβS. ADPβS is a ligand claimed to be specific for P2Y$_1$ receptors, but Michel and coworkers have shown that ADP also interacts, equipotently with α,β-MeATP, with P2X receptors [30, 31]. The $K_i$ values of 0.05 ± 0.02 μM ($N$ = 3) and 0.12 ± 0.04 μM ($N$ = 3), respectively indicate affinities higher than that for ATPαS. On the contrary ATP, UTP, and 2MeSATP were one order of magnitude less effective agonists, with $K_i$ values of 1.84 ± 0.69 μM ($N$ = 3) for UTP, 1.96 ± 0.30 μM ($N$ = 3) for ATP, and 2.49 ± 0.59 μM ($N$ = 3) for 2MeSATP (Fig. 2B). A potency order of α,β-MeATP > ATP > 2MeSATP is typical for a P2X receptor, whereas the high potency of ADPβS to displace ATPαS from its binding site indicates the possible binding of ATPαS to a P2Y$_1$ receptor. Potent inhibition of [$^{35}$S]ATPαS binding by both α,β-MeATP and ADPβS is apparently in contrast to the single-site model for ATPαS described above. Thus, ATPαS could possibly bind both to a P2X- and a P2Y$_1$ receptor with very similar $K_d$ values, thus falsely indicating a single binding site. The assumption of two separate binding sites for ATPαS was further substantiated by the incomplete displacement of ATPαS by α,β-MeATP. Even at a concentration of 100 μM α,β-MeATP, only 70 to 80% of ATPαS could be displaced from its binding sites.

### *Binding of [$^3$H]α,β-MeATP*

The existence of a P2X receptor in lung membranes was further verified by incubation of isolated lung membranes with 3 nM [$^3$H]α,β-MeATP plus various displacing agents (Fig. 3). Membranes showed specific binding activity of

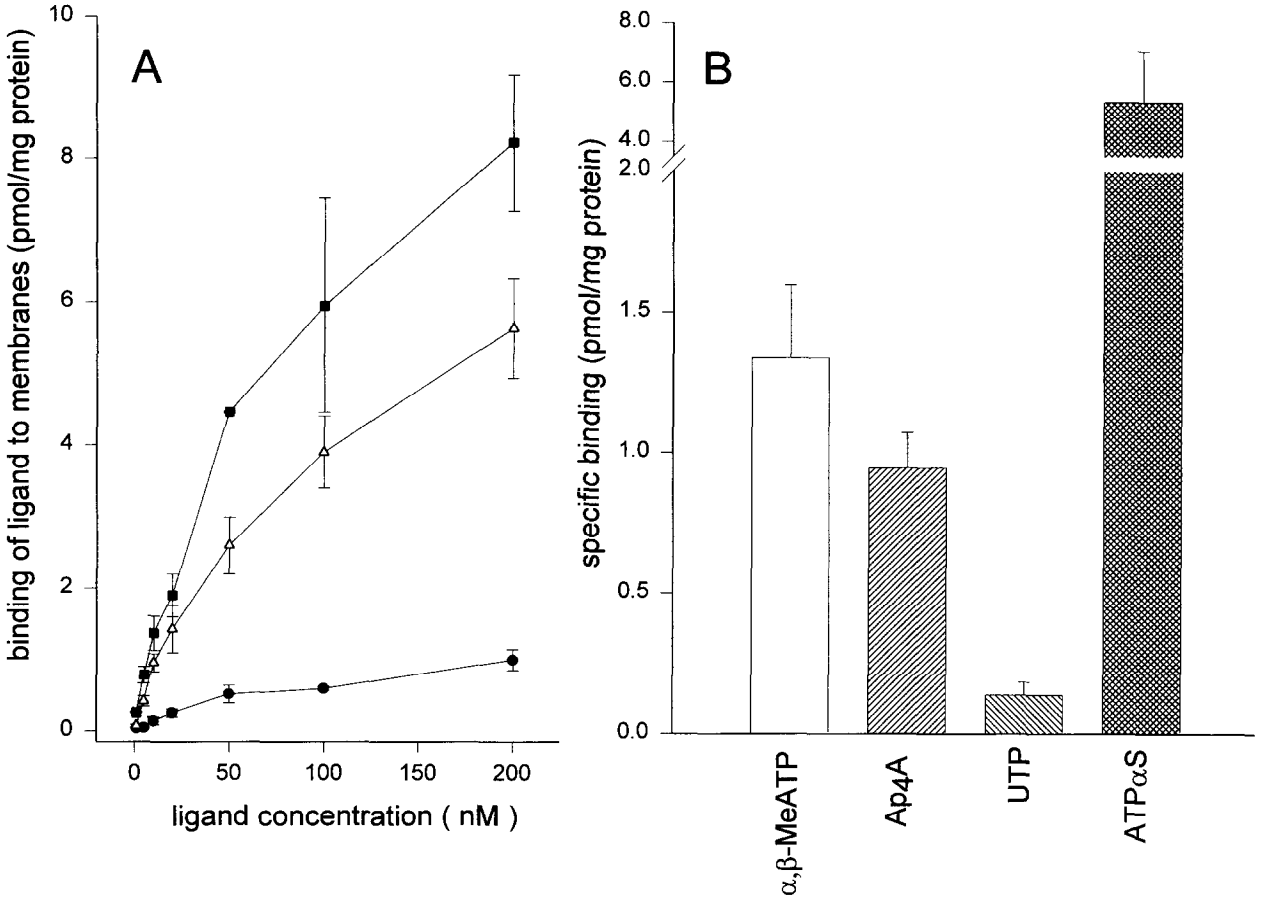


**FIG. 1. Binding of nucleotides to rat lung membranes. A) Total ligand binding was measured with increasing concentrations of radiolabelled nucleotides, nonspecific binding in the presence of the same unlabelled compound (100 μM). Specific binding given by the difference of total and nonspecific binding is shown for α,β-MeATP (■), $Ap_4A$ (△), and UTP (●). Points represent mean values obtained from 3 independent experiments; error bars are SD. B) Density of nucleotide binding sites on lung membranes. Membrane samples containing 25 μg of protein were assayed with 10 nM of [$^3$H]α,β-MeATP, [$^3$H]$Ap_4A$, [$^3$H]UTP, and [$^{35}$S]ATPαS in the presence or absence of 100 μM of the respective unlabelled ligand as described under "Materials and Methods." Data represent the mean ± SD obtained from 5 independent experiments.**

[$^3$H]α,β-MeATP of *ca.* 0.4 pmol/mg protein. Data obtained by competition studies with the unlabelled compound were best described by attributing the competition to a single population of [$^3$H]α,β-MeATP binding sites ($K_d$ = 56 ± 10 nM) with $B_{max}$ of 5.7 ± 1.1 pmol/mg protein ($N$ = 3). Membrane-bound [$^3$H]α,β-MeATP could be displaced from its binding site only in the presence of a high concentration of ATPαS with a $K_i$ value of ca. 5 μM ($N$ = 3). ATP caused only a partial displacement of 50%, even at concentrations as high as $10^{-4}$ M. UTP did not affect binding at concentrations up to 100 μM.

### *Characterization of the [$^3$H]$Ap_4A$ Binding Site*

At a ligand concentration of 10 nM, the diadenosine polyphosphate [$^3$H]$Ap_4A$ bound to lung membranes with a binding activity of 0.6 pmol/mg protein. The $K_d$ for the $Ap_4A$ binding site was 91 ± 9 nM. Partial characterization of this binding site showed that bound [$^3$H]$Ap_4A$ could be displaced by ATP with a $K_i$ value of approximately 0.9 μM (Fig. 4). α,β-MeATP was unable to displace [$^3$H]$Ap_4A$ at concentrations up to 10 μM.

### *Evidence for a Distinct Binding Site for UTP in Lung Membranes*

Specific binding activity of [$^3$H]UTP to lung membranes at a concentration of 5 nM was ca. 0.15 pmol/mg protein. The affinity for the UTP binding site was given by a $K_d$ of 43 ± 9 nM. Among the nucleotides tested, only the pyrimidine nucleotide UDP was able to compete with [$^3$H]UTP for its binding site, albeit very moderately (Fig. 5). Only about 65% of the receptor-bound [$^3$H]UTP was displaced by UDP at a concentration of 100 μM.

Displacement studies with ATP and ATP analogues showed that in contrast to $P2Y_2$ receptors, neither ATP nor ATPαS was able to displace UTP from this binding site (Fig. 5). 2MeSATP and α,β-MeATP also did not displace binding of [$^3$H]UTP. On the other hand, at high concentrations (from 10 to 100 μM) ATP or ATP derivatives

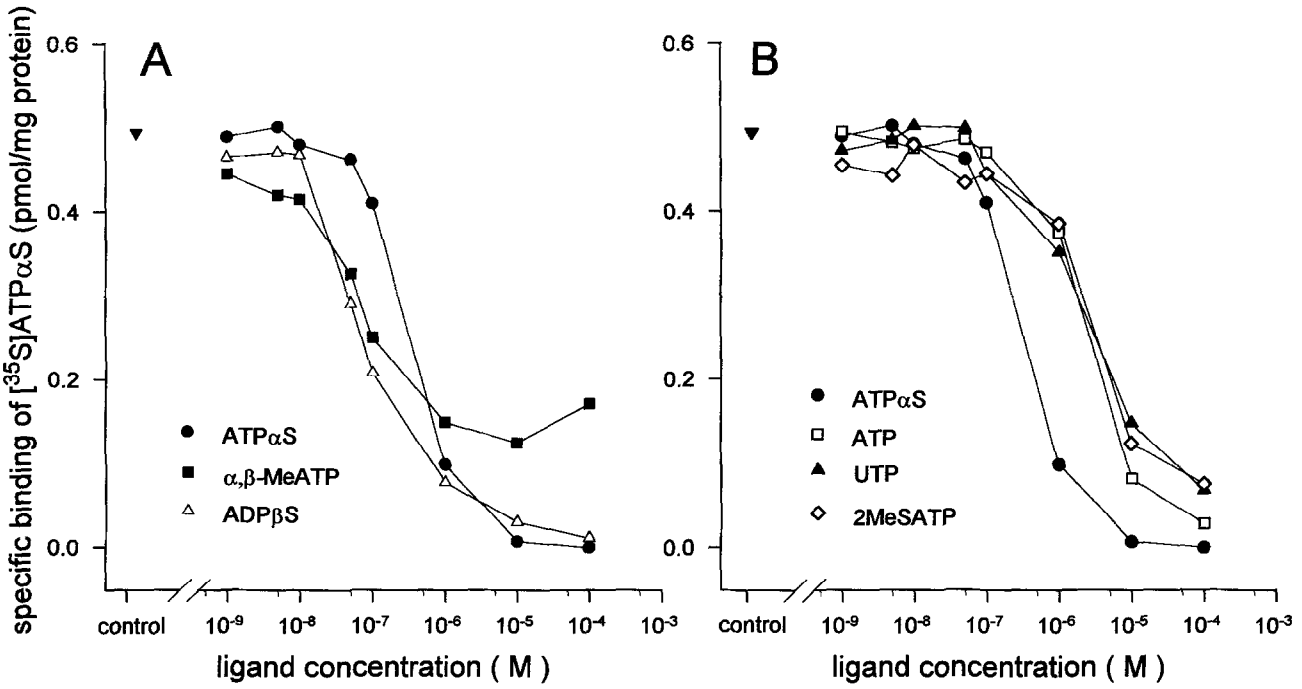


FIG. 2. Inhibition of [$^{35}$S]ATPαS binding to lung membranes by different compounds active at P2 receptors. Incubation of lung membranes (25 μg of protein) was carried out with 1 nM [$^{35}$S]ATPαS as described under "Materials and Methods." For clarity, the results obtained in the same experiment are plotted separately for ligands with high-(A) and with low-(B) affinity to the ATPαS binding site together with the displacement curve for ATPαS (●) serving as a reference for both A and B. The competitors α,β-MeATP (■), ADPβS (△), ATP (□), UTP (▲), and 2MeSATP (◇) were present in concentrations ranging from 1 nM to 100 μM. The data shown from one experiment performed in duplicate are typical of 3 independent experiments.

seemed to have a slightly stimulatory effect on the binding of UTP. In the presence of 10 μM ATPαS, the amount of UTP binding rose to values *ca.* 2.3 times the control level. This potentiation was seen to a lower degree with the other nucleotides tested.

## DISCUSSION

Submicromolar concentrations of extracellular purine and pyrimidine nucleotides induce significant regulatory effects in a variety of cells and tissues by binding to their membrane-bound receptor molecules [2, 3, 5]. Treatment of cystic fibrosis patients with the pyrimidine nucleotide UTP may result in a new therapy for this disease. In these patients, mutations in the gene for the cystic fibrosis transmembrane regulator (CFTR) were found to lead to a functionally inactive CFTR protein which is unable to regulate $Cl^-$ secretion across lung membranes [25]. The functional defect of the CFTR $Cl^-$ channel can be restored by the stimulation of an epithelial $Cl^-$ transport through application of extracellular nucleotides such as ATP or UTP, which act via purinergic receptors [4]. In recent years, an increasing number of such P2 receptors have been described [9, 10].

The pyrimidine nucleotide UTP activates receptors of the $P_{2U}$ subtype (recently designated $P2Y_2$ [1]). There have been a number of studies investigating the effects of UTP on various biological processes such as regulation of phospholipase C [2], mobilization of $Ca^{2+}$ [3], and the control of synaptic currents in the nervous system [6]. It has been possible to clone and heterogeneously express UTP receptors [11, 17, 18, 24], but until now the isolation of a native $P2Y_2$ receptor has not been described. However, the characterization of the native UTP receptor may provide a better understanding of signalling pathways. Moreover, an accurate knowledge of its ligand binding characteristics may reveal novel avenues for the treatment of lung diseases such as cystic fibrosis by the design of therapeutically effective UTP analogues, which are less susceptible than UTP to hydrolysis by nucleotidases and phosphatases present in the human airways.

### *Diversity of Nucleotide Binding Sites in Lung Membranes*

The present type of study analyzing binding sites for nucleotides in lung membranes will not enable us to unequivocally prove or disprove the existence of certain types of P2 receptors. This will only be possible in combination with the relevant physiological experiments. Past experience has already shown that evidence obtained for identity of binding sites and purinoceptors turned out to be erroneous [32, 33]. This caveat will certainly apply to ATPαS binding sites determined here, because the relatively high $B_{max}$ value shows that they include several different proteins (see "Results"). Pitfalls in the analysis of

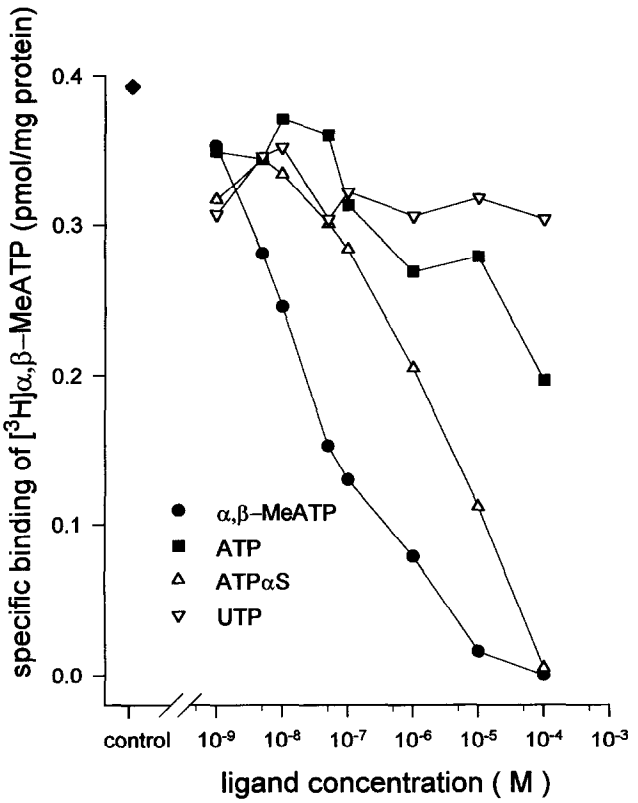


FIG. 3. Displacement of [$^3$H]α,β-MeATP binding from lung membranes by various nucleotides. Lung membranes (25 μg of protein) were incubated with 3 nM [$^3$H]α,β-MeATP as described under "Materials and Methods." The other nucleotides, α,β-MeATP (●), ATP (■), ATPαS (△), and UTP (▽) were present at the concentrations indicated. The data shown are duplicates, representing one experiment out of a total of 2 independent experiments giving similar results.

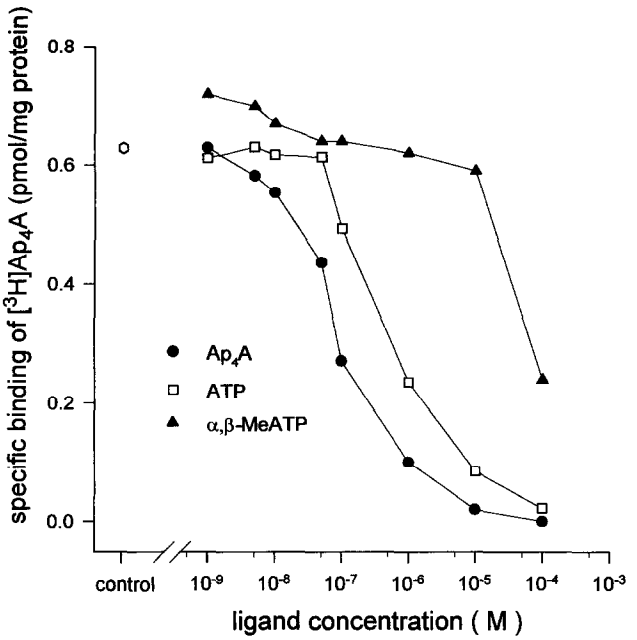


FIG. 4. Inhibition of specific binding of [$^3$H]$Ap_4A$ to lung membranes by purinergic ligands. Each ligand indicated was added together with 10 nM [$^3$H]$Ap_4A$ to a preparation of lung membranes containing 25 μg of protein. The nucleotides $Ap_4A$ (●), ATP (□), and α,β-MeATP (▲) were present at the concentrations indicated. The data shown are duplicates, representative of 2 independent experiments.

P2 receptor radioligand binding studies have also been described in [34, 35]. However, from our present experiments we are able to deduce densities and affinities of nucleotide binding sites competing for therapeutically applied nucleotides. The nature of these binding sites and their relation to P2 receptors will have to be determined.

As a prerequisite for the intended isolation of the UTP receptor, we investigated the binding sites for UTP and various purine nucleotides in lung membranes. As preparations of lung contain different cellular tissues including high amounts of blood vessels, the existence of different P2 receptors in lung membrane preparations was expected. In the present study, we could demonstrate the existence of 4 different binding sites with P2 receptor-like properties in lung membranes. Competition of [$^{35}$S]ATPαS by various ATP analogues indicates the presence of a P2X- and a $P2Y_1$-like receptor in rat lung membranes. Analysis of binding gave only one binding site for [$^{35}$S]ATPαS with a $K_i$ value of 244 nM. Competition experiments, however, indicate distinct binding sites, probably with very similar $K_d$ values for the ATPαS ligand, since both α,β-MeATP and ADPβS showed high affinity for the [$^{35}$S]ATPαS binding sites. α,β-MeATP is a very potent agonist at P2X receptors and ADPβS a potent agonist at some $P2Y_1$ receptors [9, 10]. Furthermore, the relatively high amount of binding sites (164.7 pmol/mg protein) in comparison to that for the other nucleotides applied is consistent with specific binding to several types of P2 receptors.

However, we still have to be cautious about definitively identifying the binding sites described here as distinct purinoceptors using a limited number of compounds (see for example the high affinity of 2MeSATP for $P2X_1$ and $P2X_2$ receptors [30]). Moreover, the question still arises as to which nonreceptor proteins, such as enzymes or transporters, contribute to the high amount of [$^{35}$S]ATPαS binding found with lung membrane vesicles.

Evidence for the existence of a P2X receptor in rat lung was provided by application of [$^3$H]α,β-MeATP as a ligand. This nucleotide analogue shows high selectivity for several P2X subtypes [9, 10]. Our investigation revealed a fairly high amount of binding sites for [$^3$H]α,β-MeATP on lung membranes with high affinity ($K_d$ = 56.5 nM).

Like ATP, diadenosine polyphosphates were also suggested to act as extracellular regulators. In PC 12 cells, ATP and $Ap_4A$ elicited an influx of extracellular $Ca^{2+}$ [36], but both the sensitivity of the response and the flux profile were different, suggesting the presence of two distinct P2 receptors. In rat brain synaptosomes from hippocampus, an indication for the existence of a separate purinergic receptor responding to $Ap_4A$ ($P_{2D}$, now called $P2Y_{Ap4A}$ [1]) was found, linked to the activation of protein kinase C [37]. In contrast to rat brain synaptic terminals which have binding

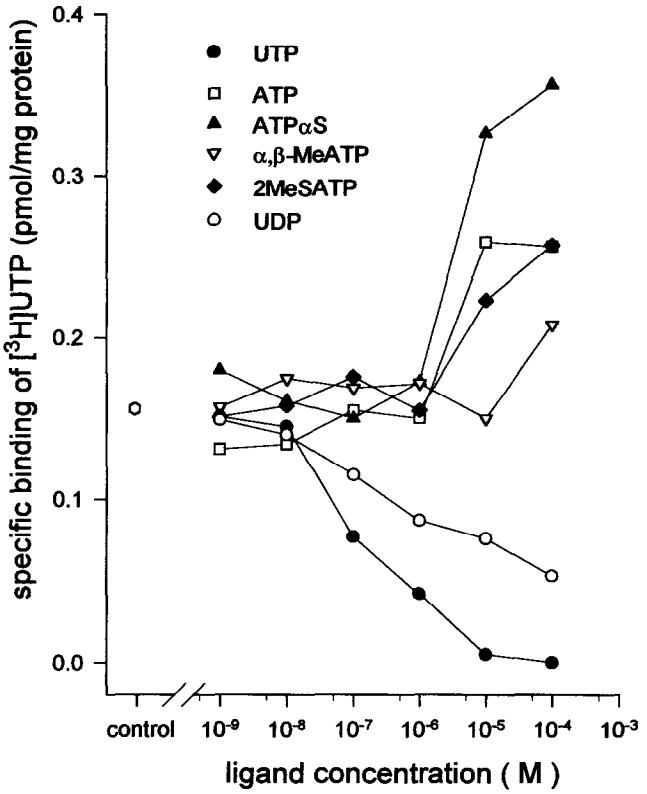


**FIG. 5. [$^3$H]UTP binding to lung membranes in the presence of various nucleotide ligands. Lung membranes (50 μg of protein) were incubated with 10 nM [$^3$H]UTP and the ligands indicated: UTP (●), ATP (□), ATPαS (▲), α,β-MeATP (▽), 2MeSATP (◆), and UDP (○). The data shown are derived from one experiment performed in duplicate and typical of 2 independent experiments.**

sites with two different affinities for $Ap_4A$ with $K_d$ values of 0.1 nM and 0.57 μM [21], we could only find one high-affinity binding site for $Ap_4A$ in lung membranes with a $K_d$ value of 91 nM. Further investigations are in progress to elucidate the question as to whether there is a population of binding sites specific for $Ap_4A$ in lung membranes or whether $Ap_4A$ binds to a P2Y receptor as in adrenal chromaffin cells [38].

### *Pyrimidine Nucleotide Receptors in Lung Membranes*

UTP has been reported to be a physiologically important extracellular signalling molecule. Until now, however, there has been ongoing controversy over whether UTP and ATP act at the same receptor or at distinct purinergic and pyrimidinergic receptors giving the same functional response. Indeed, in the last few years, a number of examples have been described where ATP or UTP elicited the same functional response by binding to a common recognition site, such as stimulation of surfactant secretion in type II cells [23], inhibition of arginine vasopressin (AVP)-stimulated osmotic water permeability in inner medullary collecting ducts of rat [39], stimulation of inositoltrisphosphate formation in rat renal mesangial cells [40], or rapid accumulation of inositol phosphates in the human airway epithelial cell line CF/T43 [2]. The cell surface receptors binding both UTP and ATP equipotently, which can be distinguished from P2X and $P2Y_1$ receptors, having a potency order of UTP = ATP > ATPγS > ADP > 2MeSATP > α,β-MeATP [10, 23], are now called $P2Y_2$ receptors. G protein-coupled $P2Y_2$ receptors were already cloned from mouse neuroblastoma cells [17], human airway epithelial (CF/T43) cells [24], and alveolae type II cells from rat [41].

In recent years, however, an increasing number of UTP receptors which do not fit the classification of $P2Y_2$ receptors but are supposed to represent a distinct class of so-called "pyrimidine receptors" have been described. In cardiac endothelial cells from guinea-pig, evidence for a distinct UTP receptor which showed no affinity for purine nucleotides was found [20]. Data obtained from C6-2B rat glioma cells also revealed the existence of a novel uridine nucleotide receptor coupled to phospholipase C that is not activated by adenosine nucleotides [19]. A phospholipase C-activating P2 receptor belonging to the G protein-coupled P2 receptor family was cloned and expressed in C6 rat glioma cells [18]. This novel P2 receptor was coupled to phospholipase C and $Ca^{2+}$ mobilization, showing a rank order of agonist potency of UTP > ADP = 2 MeSATP > ADPβS > ATP = ATPγS. Apart from the fact that UTP was the most potent agonist, the rank order of potencies for this receptor is quite different from that of $P2Y_2$ receptors.

In rat lung membranes, we have demonstrated the existence of specific binding sites for [$^3$H]UTP. However, the density of these binding sites was rather low compared with that found for the other radiolabelled nucleotides (Fig. 1B), and there was a substantial nonspecific binding of [$^3$H]UTP to the membranes, complicating interpretation of the results. Displacement studies showed that UTP was bound very strongly to its recognition site, and that UTP and ATP did not compete for a common binding site. On the contrary, at high concentrations (100 μM) ATP and ATP analogues even had a clearly stimulating effect on the binding of UTP. A possible explanation for this effect could be an allosteric low-affinity binding of ATP and its analogues, changing the binding behaviour of the receptor to UTP. On the other hand, the huge excess of other nucleotides compared to UTP might possibly prevent degradation of [$^3$H]UTP by nucleotide cleaving enzymes, still residually active in the preparation. UDP could displace [$^3$H]UTP from its binding site. In contrast to other pyrimidine nucleotide receptors, where UDP was a more potent agonist than UTP [19], in our case UDP was only a moderately active displacing agent. Thus, our results are in accordance with the existence of a UTP receptor in rat lung membranes that is distinct from $P2Y_2$ receptors previously described.

The lack of a $P2Y_2$ receptor in rat lung tissue is in contrast to results obtained from physiological experiments with rat tracheal epithelial cell monolayers, where a $P2Y_2$ receptor was found [4]. Receptors for UTP were also found

in the pulmonary vascular bed [42]. For our experiments, the large tracheae were removed before membrane preparation. Thus, localization of $P2Y_2$ receptors could be restricted to particular parts of the lung. Indeed, there is evidence that multiple populations of P2 receptors involved in the regulation of $Cl^-$ channels are located in different domains of rat lung cells [4]. In rat tracheal epithelial monolayers in primary culture, it was shown that ATP stimulated $Cl^-$ secretion from the apical or basolateral side of the monolayer. Stimulation of $Cl^-$ secretion by apical ATP was via $P2Y_2$ receptor, whereas at the basolateral side ATP stimulated several P2 receptor subtypes, including a novel type of receptor, called $P2Y_3$ [4].

The isolation and subsequent characterization of native UTP receptor binding sites from rat lung membranes which differ from $P2Y_2$ receptors may provide a better understanding of signalling pathways. Further investigations are necessary to determine how this high-affinity UTP binding protein will affect the therapeutic targets for improving $Cl^-$ secretion in CF patients by treatment with UTP analogues.

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