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Evidence for a G protein-coupled diadenosine-5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) receptor binding site in lung membranes from rat

Werner Laubinger, Georg Reiser *

Otto-von-Guericke-Universität Magdeburg, Medizinische Fakultät, Institut für Neurobiochemie, Leipziger Str. 44, D-39120 Magdeburg, Germany

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Abstract

Nucleotide receptors are of considerable importance in the treatment of lung diseases, such as cystic fibrosis. Because diadenosine polyphosphates may also be of significance as signalling molecules in lung, as they are in a variety of tissues, in the present work we investigated the binding sites for [^{3}H]diadenosine-5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) in plasma membranes from rat lung and studied their possible coupling to G proteins. We present evidence for a single high-affinity binding site for [^{3}H]Ap_4A with similar affinity for other diadenosine polyphosphates Ap_nA ($n = 2$ to 6). Displacement studies with different nucleotides revealed that the [^{3}H]Ap_4A binding site was different from P2X and $P2Y_2$ receptor binding sites. Pretreatment of lung membranes with GTPγS or GTP in the presence of Mg^{2+} increased the K_i for Ap_4A from 91 nM to 5.1 μM, which is indicative of G protein coupling. The putative coupling to G proteins was further confirmed by the enhancement of [^{35}S]GTPγS binding (to G_α proteins) to lung membranes by Ap_4A (63% increase over basal) in a concentration-dependent manner. Therefore, our data for the first time provide evidence of a G protein-coupled Ap_4A binding site in lung membranes.

Keywords: Cystic fibrosis; G protein; Lung membrane; Nucleotide; Signal transduction

1. Introduction

Purine and pyrimidine nucleotides, which occur at extremely low concentrations in the extracellular milieu act as signalling molecules in a variety of tissues (Dubyak and el Moatassim, 1993). Over the last years, in addition to ATP and UTP a number of unusual nucleotides, such as diadenosine polyphosphates, have also been found to play a role in signal transduction (Ogilvie et al., 1996). Diadenosine-5′,5‴-P^1,P^4-tetraphosphate (Ap_4A), one representative of this type of nucleotide, is implicated in the initiation of DNA replication (Weinmann-Dorsch and Grummt, 1986) and in the processing of ADP-ribosylated histones (Surowy and Berger, 1983). Diadenosine polyphosphate nucleotides can be detected also in the secretory granules of chromaffin cells (Pintor et al., 1992), neuronal synaptic granules (Pintor et al., 1993), and platelet granules (Zamecnik, 1983). They are also reported to act as extracellular signalling molecules. After the release of diadenosine polyphosphates into the extracellular environment, these nucleotides exert effects on different cell types, such as activation of glycogen phosphorylase in isolated liver cells (Craik et al., 1993), physiological control of blood pressure (Schlüter et al., 1994), and activation of gluconeogenesis in rat proximal tubules (Edgecombe et al., 1997). In the lung, however, where nucleotides play an important physiological role as regulators of epithelial ion transport processes, the existence of receptors specific for diadenosine polyphosphates has not yet been investigated.

Receptors that bind ATP and/or ADP are classified as P2 receptors. According to their pharmacological response to ATP analogues, P2 receptors are subclassified into two distinct families, ligand-gated ion channels (P2X) and G protein-coupled receptors (P2Y) (Abbracchio and Burnstock, 1994; Barnard et al., 1994). One member of the P2Y family ($P2Y_2$) is also activated by the pyrimidine nucleotide UTP and by the purine nucleotide ATP (O'Connor, 1992). Other members of this family ($P2Y_4$ and $P2Y_6$)

* Corresponding author. Tel.: +49-391-6713088; Fax: +49-391-6713097; E-mail: georg.reiser@medizin.uni-magdeburg.de

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are specific pyrimidine nucleotide receptors (Communi et al., 1996a,b). The DNA sequences of some of these receptors have already been elucidated and the corresponding receptor proteins expressed in heterologous systems (Lustig et al., 1993; Parr et al., 1994; Rice et al., 1995). However, despite intensive efforts over the last few years the question is still completely open as to whether there is a receptor common for both ATP and the diadenosine polyphosphates or whether there are distinct receptors for Ap_4A. Pharmacological studies using the cloned human $P2Y_2$ receptor (Lazarowski et al., 1995) indicate that Ap_4A binds to the same type of receptor as ATP or UTP, but there is also clear evidence for a distinct Ap_4A receptor (called $P2_{Ap4A}$ or P_{2D}) in the nervous system (Hilderman et al., 1991; Pintor et al., 1993; Pintor and Miras-Portugal, 1995a,b). A purinoceptor in central synapses that recognized specifically Ap_4A was not activated by ATP and synthetic ATP analogues (Pintor et al., 1995). Recently it was shown that diadenosine polyphosphates had a bimodal effect on [^{35}S]ATPαS binding in synaptosomal membranes from rat brain cortex (Schäfer and Reiser, 1997), supporting the existence of separate receptors specific for diadenosine polyphosphates.

In airway tissue there are various Cl^- channels which are activated by different signalling pathways: (i) cAMP-stimulated Cl^- transport mediated by the cystic fibrosis transmembrane conductance regulator Cl^- channel and (ii) an alternative Cl^- transport coupled to intracellular Ca^{2+} mobilization after activation of P2 receptors (Hwang et al., 1996). The latter pathway plays an important role in the inheritable disease cystic fibrosis, where Cl^- secretion by the cystic fibrosis transmembrane conductance regulator Cl^- channel is impaired (Jiang et al., 1993). Nucleotides that activate P2 receptors are a promising tool in the treatment of cystic fibrosis. In clinical studies the pyrimidine nucleotide UTP was successfully applied as a therapeutic agent (Olivier et al., 1996). But as UTP is very unstable under the conditions existing in the cystic fibrosis lung, it would be advantageous to find a more stable natural or synthetic nucleotide analogue that can replace UTP as a $P2Y_2$ receptor agonist. Thus, the relatively long half-life of Ap_4A in vivo makes investigation of the physiological effects of this compound in lung tissue an important issue. For assessment of therapeutic strategies using UTP analogues it will be necessary to know whether Ap_4A interacts with UTP receptors or with distinct receptors in lung membranes. Two distinct P2Y receptors have been identified in human tracheal gland serous cells (Merten et al., 1998).

In experiments with human bronchial epithelial cells (cell line HBE-1), cytosolic [Ca^{2+}] rose after stimulation with ATP, UTP or Ap_4A (Ubl and Reiser; unpublished data). The response evoked by Ap_4A was different from that evoked by ATP or UTP and no heterologous desensitization of the Ca^{2+} response was found in the airway epithelial cells. These results for human lung epithelial cells make the characteristics of specific Ap_4A receptors in the lung an interesting issue for further physiological studies. In previous experiments we found evidence for the existence of several types of P2 receptor-like binding sites in membranes from rat lung, one of which bound [^{3}H]Ap_4A specifically (Laubinger and Reiser, 1998). Therefore, in the present work we characterized the properties of Ap_4A receptor binding sites in lung membranes from rats. In displacement experiments we can distinguish the Ap_4A binding site from other P2 receptors (P2X and $P2Y_2$). Furthermore, we demonstrate by stimulation of [^{35}S]GTPγS binding that the Ap_4A binding site is coupled to G proteins. Thus, for the first time there is clear evidence for the existence of an Ap_4A binding site in lung membranes that is coupled to G proteins.

2. Materials and methods

2.1. Materials

ATP, α,β-methyleneadenosine 5′-triphosphate (α,β-MeATP), adenosine 5′-*O*-(2-thio)diphosphate (ADPβS), diadenosine 5′,5‴-P^1,P^2-pyrophosphate (Ap_2A), diadenosine 5′,5‴-P^1,P^3-triphosphate (Ap_3A), Ap_4A, diadenosine 5′,5‴-P^1,P^5-pentaphosphate (Ap_5A), diadenosine 5′,5‴-P^1,P^6-hexaphosphate (Ap_6A), GTP, guanosine 5′-*O*-(3-thio)triphosphate (GTPγS), GDP and UTP were from Sigma (Deisenhofen, Germany). [^{3}H]Ap_4A (11.4 Ci/mmol; 422 GBq/mmol) was from Amersham (Braunschweig, Germany) and [^{35}S]GTPγS (1250 Ci/mmol; 46.3 TBq/mmol) was from NEN (Brussels, Belgium).

2.2. Preparation of lung membranes

Lungs dissected from 12 rats (*Rattus rattus*) were washed with sufficient amounts of ice-cold phosphate-buffered saline (137 mM NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4, and 1.4 mM KH_2PO_4, pH 7.4). Receptor-containing plasma membranes were prepared as described earlier (Laubinger and Reiser, 1998). The protein content was determined according to Lowry et al. (1951), using bovine serum albumin as a standard.

2.3. [^{3}H]Ap_4a binding experiments

Binding of [^{3}H]Ap_4A was measured by incubation of lung membranes (50 μg of protein) in incubation medium (final volume 0.2 ml) containing 25 mM HEPES, pH 7.4, 50 mM NaCl, and 5 mM KCl (binding buffer) for 35 min at 4°C. Under these conditions, the binding of radiolabelled nucleotides reached equilibrium and remained nearly constant for up to 60 min of incubation. For competition binding experiments the binding of a constant amount of the labelled nucleotide was determined in the presence of

increasing concentrations of various unlabelled nucleotides. After incubation, the 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 s) with 300 μl of binding buffer and resuspended in 100 μl of 1.5% (w/v) sodium dodecyl sulfate. Membrane-bound radioactivity was measured in 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.

2.4. [^{35}S]GTPγS binding assay

The binding assay was performed as described for [^{3}H]Ap_4A binding with the following modifications. The binding buffer (10 mM HEPES, pH 7.4, 100 mM NaCl and 10 mM $MgCl_2$) contained 140 pM [^{35}S]GTPγS, 0.1 μM GDP and the appropriate amount of Ap_2A, Ap_4A, or Ap_5A in a total volume of 0.1 ml. The reaction, carried out according to a standard protocol (Befort et al., 1996; Akam et al., 1997), was started by the addition of lung membrane preparation (10 μg of protein) and was continued for 45 min at 30°C. The reaction was stopped and the pellets were washed twice with 150 μl of binding buffer and analyzed as described above. Nonspecific binding was determined in the presence of 10 μM GTPγS.

2.5. Data analysis

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

2.6. High-performance liquid chromatography (HPLC) analysis

The stability of Ap_4A under the test conditions used was analyzed by HPLC. After experimental incubation 200 μl of the complete binding assay mixture with the appropriate amount of Ap_4A was mixed with 50 μl of 25% (w/v) trichloroacetic acid. After 10 min on ice, proteins were precipitated by centrifugation (25,000 g, 12 min, 4°C). Trichloroacetic acid was removed from the supernatant by 4 successive extractions with 500 μl diethylether. After evaporation of the remaining traces of diethylether (60°C), the samples were diluted to 750 μl with H_2O. Nucleotides were separated by Resource Q ion exchange chromatography on a HPLC (Kontron, Neufahrn/Obb., Germany). The column was run with 25 mM HEPES pH 7.4 containing 50 mM NaCl. Samples (200 μl) prepared as described above were injected onto the column and eluted using a linear gradient of NaCl (50–750 mM) in 25 mM HEPES pH 7.4. Elution profiles were analyzed by monitoring absorption at 254 nm. Authentic compounds (200 μl of a 5 μM solution in H_2O) were used to identify the resulting elution peaks.

3. Results

3.1. Evidence for a specific Ap_4A binding site in rat lung

Lung membrane preparations from rat were incubated with 10 nM [^{3}H]Ap_4A in the presence and absence of unlabelled Ap_4A at 4°C. The incubation was stopped at various times by centrifugation and subsequent washing of the membranes. This collecting and washing procedure prolonged the incubation for an additional 15 min. Maximal binding of [^{3}H]Ap_4A to lung membranes was attained after approximately 50 min and remained constant for at least 75 min (Fig. 1A). To determine the density of the Ap_4A binding sites, we incubated lung membranes with

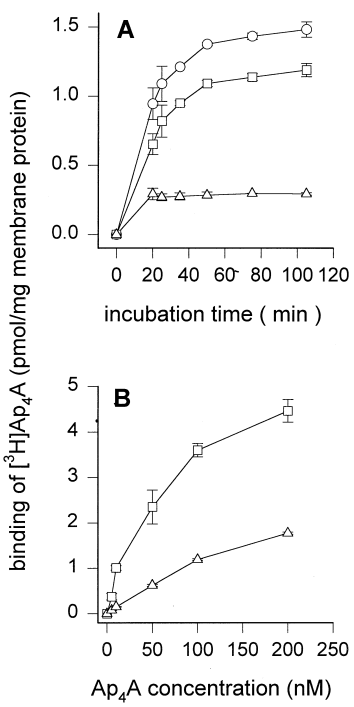

Fig. 1. Characteristics of Ap_4A binding. (A) Time course of [^{3}H]Ap_4A binding to rat lung membranes. Samples of lung membranes containing 50 μg of protein were incubated with 10 nM [^{3}H]Ap_4A on ice. The incubation was stopped and the incubated membranes were treated as described under materials and methods. The time needed for collecting and washing the membranes was added to the incubation time on ice, giving the total incubation time shown. Total binding (○) was measured in the absence and nonspecific binding (△) in the presence of 100 μM unlabelled Ap_4A. The difference between total and nonspecific binding represents the specific binding (□). Results are mean values from three independent experiments. (B) Binding of Ap_4A to rat lung membranes. Lung membranes (50 μg of protein) were incubated with increasing concentrations of radiolabelled [^{3}H]Ap_4A as described under materials and methods. Specific binding (□) represents the difference between total binding and nonspecific binding in the presence of 100 μM unlabelled Ap_4A (△). Points represent mean values from three independent experiments. Error bars indicate S.D. Where error bars are not shown, they are smaller than the symbol used.

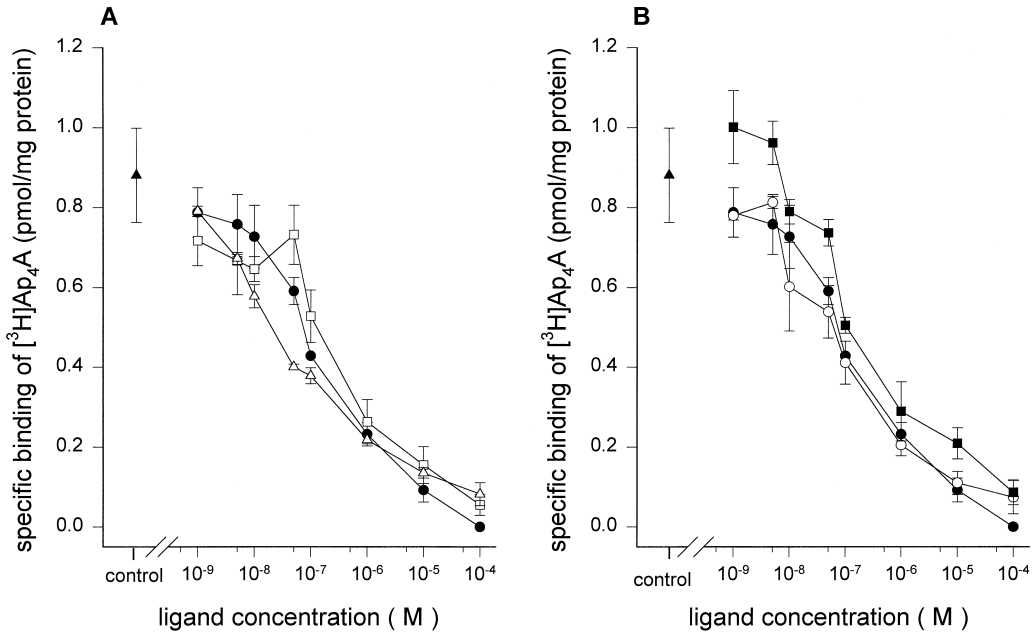

Fig. 2. Displacement of [^{3}H]Ap_4A from lung membranes by diadenosine polyphosphate nucleotides. Lung membranes (50 μg of protein) were incubated with 10 nM [^{3}H]Ap_4A (▲) as described under materials and methods. For clarity, the results obtained in the same experiment are plotted separately for less (A) and more phosphorylated (B) homologues of diadenosine polyphosphates with the displacement curve for Ap_4A (●) serving as a reference for both (A) and (B). The competitors Ap_2A (△), Ap_3A (□), Ap_5A (○), and Ap_6A (■) were present in concentrations ranging from 1 nM to 100 μM. The data shown are mean values ± S.D. from three independent experiments.

radioactively labelled [^{3}H]Ap_4A at concentrations ranging from 5 to 200 nM (Fig. 1B). The binding data from saturation studies were analyzed by using nonlinear regression models assessing the existence of one or two binding sites. This analysis yielded a single high-affinity binding site for Ap_4A with a K_d value of 91.2 ± 8.8 nM ($n = 6$) and a maximal binding capacity, B_{max}, of 6.9 ± 1.6 pmol/mg of protein. Nonspecific binding in the presence of 100 μM unlabelled Ap_4A was less than 30% of the total binding. [^{3}H]Ap_4A was displaced by unlabelled Ap_4A with a K_i value of 91.0 ± 9 nM, a value almost identical to the K_d value.

The stability of the ligands applied is of paramount importance in binding assays performed to study receptors. As potential breakdown products of Ap_4A such as ATP, ADP, and AMP can exert effects on a range of purinoceptors which are different from the nucleotide binding site studied here, we examined whether such degradation products arise during incubation of lung membranes with Ap_4A. By HPLC analysis Ap_4A can be clearly separated from the possible degradation products by its different retention time (R_t). After incubation with membranes, Ap_4A (R_t = 8.3 min) was the only nucleotide found in the assay mixture, and no significant amounts of ATP (R_t = 7.8 min), ADP (R_t = 7.1 min), or AMP (R_t = 5.1 min) were detected (less than 1%). Thus, the effects described here can be attributed to the intact ligand. This is in good agreement with the findings of other workers showing a high stability of diadenosine polyphosphates (Lazarowski et al., 1995; Edgecombe et al., 1997).

3.2. Ligand specificity 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

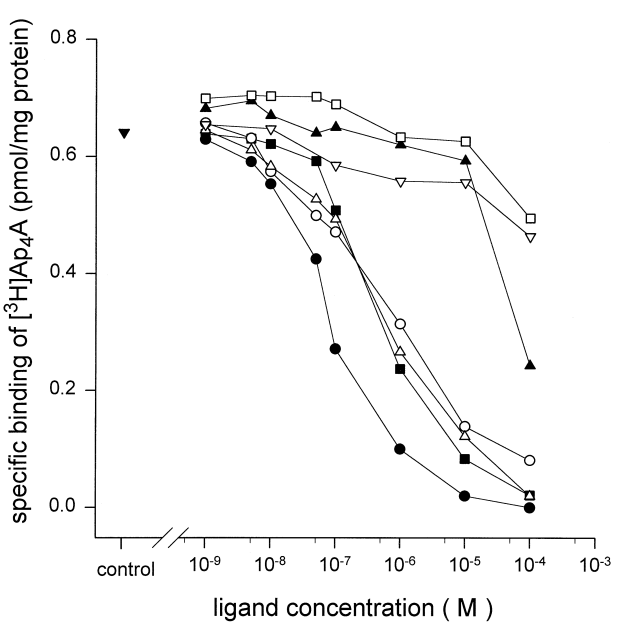

Fig. 3. [^{3}H]Ap_4A binding to lung membranes in the presence of various nucleotide ligands. Lung membranes containing 50 μg of protein were incubated with 10 nM [^{3}H]Ap_4A (▼) and increasing concentrations of the ligands indicated: Ap_4A (●), ATP (■), ATPαS (△), ADPβS (○), α,β-MeATP (▲), UTP (□), and GTPγS (▽). The data shown were derived from one experiment performed in duplicate and are typical of three independent experiments giving similar results.

specific binding activity of about 0.9 pmol/mg protein. The specificity of the [^{3}H]Ap_4A binding site was investigated by using different diadenosine polyphosphates (Fig. 2). Both the less phosphorylated homologue Ap_2A (Fig. 2A) and the more phosphorylated homologue Ap_5A (Fig. 2B) were able to displace [^{3}H]Ap_4A in a way very similar to that observed with unlabelled Ap_4A. Ap_3A and Ap_6A, however, were slightly less effective than unlabelled Ap_4A in displacing [^{3}H]Ap_4A from its binding site.

[^{3}H]Ap_4A was also displaced by some adenine mononucleotides known to be potent agonists of P2 receptors (Fig. 3). However, with K_i values of approximately 0.9 ± 0.4 μM for ATP ($n = 3$), 1.3 ± 0.7 μM for ATPαS ($n = 3$), and 0.7 ± 0.2 μM for ADPβS ($n = 3$) the affinity of these nucleotides for the binding site was about 10 times lower than that of Ap_4A. α,β-MeATP, a potent agonist of some P2X receptors, only partially displaced [^{3}H]Ap_4A from its binding site at relatively high concentrations (above 100 μM). Both, the pyrimidine nucleotide UTP and GTPγS, a guanine nucleotide, which binds to the α-subunit of G proteins were unable to displace [^{3}H]Ap_4A in concentrations up to 100 μM.

3.3. Effect of GTP and GTPγS on the binding of [^{3}H]Ap_4A

As the signal transduction of P2Y receptors is mediated by G proteins, the influence of GTP on the binding of [^{3}H]Ap_4A to lung membranes was investigated. Since these experiments were performed in the presence of millimolar concentrations of Mg^{2+}, the effect of Mg^{2+} on binding affinity was also determined. Experiments in the presence of 5 mM Mg^{2+} showed that Mg^{2+} had no effect on [^{3}H]Ap_4A binding over the range of ligand concentrations tested (data not shown).

Preincubation of lung membranes with 10 μM GTP or the hydrolysis-resistant GTP analogue GTPγS in the presence of 5 mM $MgCl_2$ had a striking effect on the binding of Ap_4A (Fig. 4A). The maximal binding of the radioactive ligand at the concentration used (10 nM) was reduced by 50% because the affinity for Ap_4A was strongly decreased after preincubation with Mg^{2+} GTPγS. The K_i value was increased 57-fold from 91 nM to 5.1 ± 0.9 μM ($n = 3$). The displacement curve was shallow, extending over several log units. Mathematical analysis revealed that some high-affinity binding sites remained with a K_i of 36.0 ± 8.6 nM ($n = 3$), a value comparable to the K_i obtained for Ap_4A without preincubation with GTP or GTPγS (91 nM).

3.4. Ap_4A receptor-mediated binding of [^{35}S]GTPγS

Enzymatic GDP/GTP exchange is proof of ligand binding to G-protein-coupled receptors (Akam et al., 1997). The method of agonist-stimulated [^{35}S]GTPγS binding has

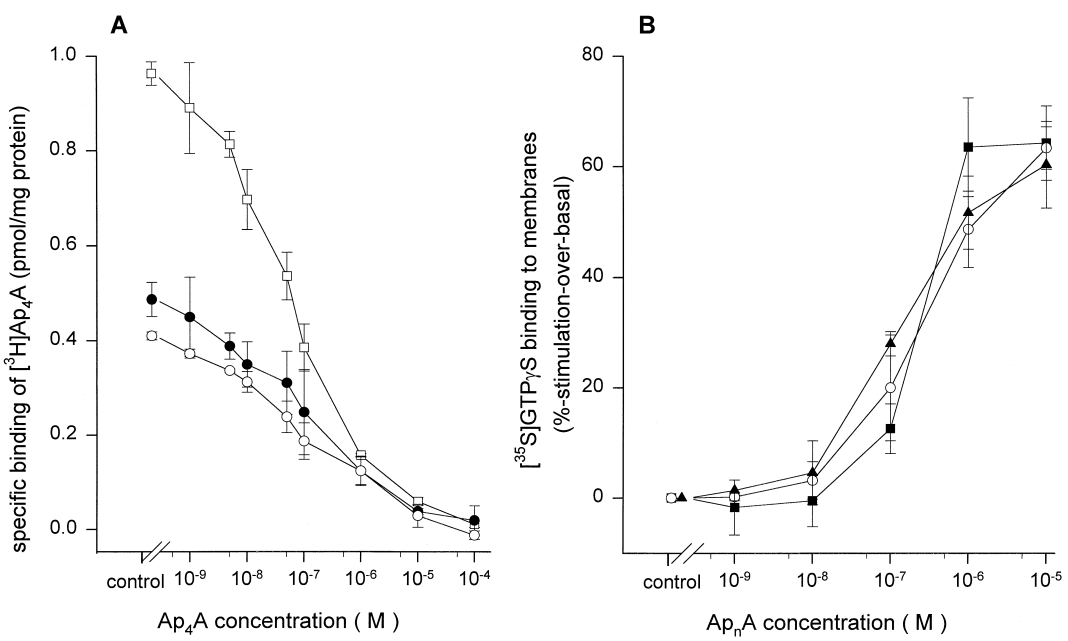

Fig. 4. Coupling of Ap_4A binding sites to G proteins. (A) Influence of GTP/GTPγS on the binding of [^{3}H]Ap_4A to lung membranes. Lung membranes containing 50 μg of protein were preincubated in the presence of 5 mM $MgCl_2$ with 10 μM GTP (●) or 10 μM GTPγS (○) or without GTP or GTPγS (□) for 30 min. For the binding reaction 10 nM [^{3}H]Ap_4A and increasing concentrations of unlabelled Ap_4A were added. Control represents binding without unlabelled Ap_4A. Data are mean values ± S.D. from triplicates of two independent experiments. (B) Diadenosine polyphosphate-stimulated [^{35}S]GTPγS binding to membranes from rat lung. Lung membranes (10 μg of protein) were incubated with 140 pM [^{35}S]GTPγS and increasing amounts of the agonists Ap_2A (○), Ap_4A (●), and Ap_5A (▲) as described under materials and methods (control is in the absence of Ap_nA). Data are expressed as % stimulation-over-basal and are means ± S.D. of five independent experiments carried out in triplicate. Nonspecific basal binding was measured in the presence of 10 μM unlabelled GTPγS. Basal [^{35}S]GTPγS binding to rat lung membranes was 0.29 ± 0.09 pmol/mg protein ($n = 15$).

been used successfully as a tool to evaluate G-protein coupled receptors (Befort et al., 1996). Therefore, in initial experiments the conditions for [^{35}S]GTPγS binding were optimized. Different concentrations of [^{35}S]GTPγS, GDP, and NaCl were used to investigate the stimulatory effects on [^{35}S]GTPγS binding to lung membranes of a saturating concentration of the P2 receptor ligand Ap_4A (100 μM).

With 140 pM [^{35}S]GTPγS, the maximal Ap_4A-stimulated [^{35}S]GTPγS binding was found at a concentration of 0.1 μM GDP, independently of a low (10 mM) or a high (100 mM) concentration of NaCl in the incubation mixture. As described in other reports (e.g., in Akam et al., 1997), the concentration of GDP was critical for [^{35}S]GTPγS binding in our experiments. GDP concentrations higher than 0.1 μM (up to 10 μM) considerably reduced [^{35}S]GTPγS binding to lung membranes. Maximal stimulation was reached after an incubation time of 45 min at 30°C (data not shown). Thus, for all further experiments rat lung membranes were incubated with 140 pM [^{35}S]GTPγS, 0.1 μM GDP, 10 mM $MgCl_2$, and 100 mM NaCl at 30°C for 45 min.

Evidence for the activation of G proteins by P2 receptors, which bind diadenosine polyphosphates, was obtained from experiments showing the effect of increasing concentrations of Ap_4A on [^{35}S]GTPγS binding to lung membranes (Fig. 4B). Ap_4A stimulated [^{35}S]GTPγS binding in a concentration-dependent manner. At a concentration of 1 μM Ap_4A the increase in [^{35}S]GTPγS binding was 63.5 ± 8.9% ($n = 5$) compared with the value in the absence of Ap_4A. The Ap_4A concentration causing half-maximal stimulation (100 nM) corresponded to the affinity of the [^{3}H]Ap_4A binding site described above ($K_d = 91$ nM). A similar stimulation was obtained when diadenosine polyphosphates with a shorter (Ap_2A) or a longer phosphate chain (Ap_5A) connecting the adenosine moieties were used.

4. Discussion

Diadenosine polyphosphates play an important role as intracellular and extracellular messenger molecules (Surowy and Berger, 1983; Zamecnik, 1983; Weinmann-Dorsch and Grummt, 1986; Pintor et al., 1992, 1993; Craik et al., 1993; Schlüter et al., 1994; Ogilvie et al., 1996; Edgecombe et al., 1997). After being released from storage granules, they can exert their effects on remote target cells, since diadenosine polyphosphates are much more stable in vivo than other nucleotides, such as ATP or UTP, that also act as signalling molecules (Lazarowski et al., 1995; Edgecombe et al., 1996). This relative stability makes them a target of interest as far as their possible application as therapeutic agents is concerned. Thus, in blood diadenosine polyphosphates are relatively long-lived molecules and exert potent vasoactive properties after intravenous injection (Schlüter et al., 1996). Furthermore, their effect on blood pressure regulation depends on the length of the phosphate chain in the diadenosine polyphosphate molecule: Ap_2A, Ap_3A, and Ap_4A lower blood pressure, whereas Ap_5A and Ap_6A increase the blood pressure.

In cystic fibrosis the nucleotide UTP is applied as therapeutic agent to stimulate Cl^- secretion by a Cl^- channel that is different from the cystic fibrosis transmembrane conductance regulator Cl^- channel which is malfunctional due to a genetic defect (Olivier et al., 1996). The pyrimidine nucleotide UTP activates a P2 receptor ($P2Y_2$) on the apical membrane of lung epithelial cells (Hwang et al., 1996). In pharmacological studies with the cloned human $P2Y_2$ receptor it was shown that Ap_4A was as potent as UTP or ATP as agonist (Lazarowski et al., 1995). In inhalation therapy for cystic fibrosis, nucleotides act as local agents that must reach their corresponding receptors on their way through the obstructed airway system which is full of hydrolytic enzymes. Thus, the considerably more stable diadenosine polyphosphates might be an option to replace the unstable nucleotide UTP as a P2 receptor agonist. But despite many investigations in the past, it is still uncertain whether Ap_4A is just another potent agonist of P2 receptors (e.g., $P2Y_2$) or whether there are distinct P2 receptor subtypes specific for Ap_4A. In the nervous system evidence was found for a distinct $P2_{Ap4A}$ receptor (Hilderman et al., 1991; Pintor et al., 1993; Pintor and Miras-Portugal, 1995a,b). Our findings that human lung epithelial cells respond to Ap_4A application with an increase in the cytosolic Ca^{2+} concentration make the question of the existence of specific Ap_4A receptors in the lung an interesting issue for further physiological studies.

In the present work we identified a high-affinity binding site for [^{3}H]Ap_4A in lung membranes. Compared with the maximal binding capacity (B_{max}) of 86 pmol/mg of protein reported for plasma membranes from rat liver and 20 pmol/mg of protein for rat proximal tubule (Edgecombe et al., 1997), the B_{max} for plasma membranes from rat lung was significantly lower (6.9 pmol/mg of protein). In lung membranes the K_d value for Ap_4A was 91 nM, whereas it was 1.76 μM in liver membranes (Edgecombe et al., 1996). In contrast to rat brain synaptic terminals, which have binding sites with two different affinities for Ap_4A (K_d 0.1 nM and 0.57 μM (Nordone and Pivorun, 1995)), we could find only one high-affinity binding site for Ap_4A in lung membranes (K_d 91 nM). There were also differences concerning the affinity for different Ap_nA homologues. In liver cell membranes from rat both less (Ap_2A, Ap_3A) and more phosphorylated (Ap_5A, Ap_6A) homologues were much less effective than unlabelled Ap_4A in displacing bound [^{3}H]Ap_4A. In rat lung membranes, however, Ap_2A and Ap_5A were as potent as Ap_4A as displacers. Ap_3A and Ap_6A were only slightly less effective than Ap_4A in displacing [^{3}H]Ap_4A from the membranes.

Thus, the binding site for [^{3}H]Ap_4A in lung membranes is less specific with regard to the length of the phosphate

bridge of its diadenosine polyphosphate nucleotide ligand than it is in liver membranes and renal cortex membranes (Edgecombe et al., 1996). Since contrasting effects on blood pressure are seen depending on the number of phosphates in the diadenosine molecule (Schlüter et al., 1996), the relative unspecificity of the [^{3}H]Ap_4A binding site in lung with regard to the length of the phosphate chain may be advantageous in possible clinical applications. Thus, based on this relative unspecificity of the binding site for a number of diadenosine polyphosphates Ap_nA ($n = 2$ to 6), the compound with the least negative side effects could be chosen for treatment of cystic fibrosis.

Ap_4A is a very effective agonist at the cloned human $P2Y_2$ receptor stably expressed in 1321N1 human astrocytoma cells (Lazarowski et al., 1995), and in rat liver membranes UTP displaces [^{3}H]Ap_4A almost as effectively as unlabelled Ap_4A (Edgecombe et al., 1996). These results suggest that either UTP recognizes the same P2Y-like receptor or Ap_4A recognizes a $P2Y_2$ receptor that is also present in the liver membranes. In rat lung membrane preparations UTP was unable to displace [^{3}H]Ap_4A from its binding site. Since UTP was able to displace [^{3}H]UTP from other P2 receptor-like binding sites in rat lung membranes under identical experimental conditions (Laubinger and Reiser, 1998), we can conclude that UTP was not degraded during the incubation. Thus, our data show no evidence for the existence of a $P2Y_2$ receptor with a common binding site for Ap_4A and UTP in rat lung membranes. It is also unlikely that [^{3}H]Ap_4A binds to a P2X receptor because α,β-MeATP, a very potent agonist for P2X receptors, had only negligible activity in displacing [^{3}H]Ap_4A binding.

For the [^{35}S]ATPαS nucleotide binding site in rat brain cortical synaptosomes, preincubation with 5 μM GTP or GTPγS increased the K_d for the high-affinity binding site five-fold (Schäfer and Reiser, 1997). Uncoupling of the G protein by binding of GTP decreased the agonist affinity for the G protein-coupled P2 receptor. This diminishing effect of GTP was also seen in physiological experiments with ATP (Boyer et al., 1989). In rat lung membranes, preincubation with GTP or GTPγS decreased the affinity of the [^{3}H]Ap_4A binding site, resulting in two distinct populations of binding sites, namely, a high-affinity site ($K_i = 36$ nM) and a low-affinity site ($K_i = 5.1$ μM), reflecting a 57-fold increase compared with the K_i of 91 nM obtained without preincubation with GTP or GTPγS. The most likely explanation for this effect is that uncoupling of the G protein by binding of GTP or GTPγS decreased the agonist affinity of the G protein-coupled receptor (Cooper et al., 1989; Simon et al., 1995).

Further evidence for the coupling of the Ap_4A binding site to G proteins was provided by the [^{35}S]GTPγS binding experiments. Under optimized conditions, these experiments with a hydrolysis-resistant GTP analogue can provide information about the extent of agonist-stimulated guanine nucleotide exchange in G proteins (Akam et al., 1997). In the presence of 1 μM Ap_4A, [^{35}S]GTPγS binding to lung membranes was stimulated by about 63% above the basal level. This extent of stimulation is comparable with that obtained by other groups investigating different receptor types, e.g., activation of G proteins by type 1α metabotropic glutamate (mGlu) receptors (Akam et al., 1997). The concentration of Ap_4A for half-maximal stimulation (EC_{50} about 100 nM) was in good accordance with the affinity value of the Ap_4A binding site (91 nM). The other diadenosine polyphosphates, such as Ap_2A or Ap_5A, also caused a stimulation of [^{35}S]GTPγS binding similar to that elicited by Ap_4A. Bearing in mind the similar affinity of the different Ap_nA for the [^{3}H]Ap_4A binding site, these results support the assumption that there is a single binding site that does not distinguish between the different Ap_nA and which is coupled to G proteins.

Our results demonstrate the existence of a receptor binding site for Ap_4A in lung membranes that is different from P2X and $P2Y_2$ receptor binding sites. This binding site, which is coupled to G proteins and which is a putative receptor for Ap_4A, could belong to the superfamily of P2Y receptors. This first evidence for the existence of a distinct Ap_4A receptor coupled to G proteins in lung will allow investigators to establish the physiological functions of diadenosine polyphosphates in the lung. Further experiments have to determine the identity of this binding site and the significance of this receptor in signal transduction.

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