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Extracellular ATP Binding Proteins as Potential Receptors in Mucociliary Epithelium: Characterization Using [^{32}P]3′-O-(4-Benzoyl)benzoyl ATP, a Photoaffinity Label

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Abstract. 3′-O-(4-benzoyl)benzoyl ATP (BzATP) was used as a photoaffinity analog of ATP to label potential ATP receptors in ciliated cells. Like ATP, without photoactivation, BzATP stimulated the ciliary beat frequency in tissue culture up to threefold. Irradiation of intact cells in the presence of [α-^{32}P]BzATP followed by SDS-PAGE and autoradiography revealed two labeled proteins with molecular masses of 46 and 96 kDa (p46 and p96). Photolabeling of both proteins was susceptible to digestion with trypsin, implying that the labeled proteins are at least partially exposed on the extracellular surface of the plasma membrane. The dependence of ^{32}P incorporation in both proteins on [α-^{32}P]BzATP concentration was similar. Labeling of p46 but not p96 required Ca^{2+} or Mg^{2+}. Various nucleotides stimulated the ciliary frequency, and inhibited the photolabeling of p46 and p96. The rank order of apparent affinity for p46 is: ATP ≅ ADP > GTPγS > ADPβS, UTP, 2MeSATP, AMP-PNP > AMP-PCP > AMP > adenosine; for p96 it is: ADP ≅ ADPβS ⩾ ATP ⩾ AMP-PCP, AMP-PNP > GTPγS ⩾ AMP > 2MeSATP, UTP, adenosine. The rank of stimulation of ciliary beat frequency is: ADPβS, UTP ⩾ 2MeSATP, GTPγS, AMP-PNP, ATP ⩾ ADP > AMP-PCP > adenosine > AMP. These results suggest the involvement of p46 in the stimulatory effect of extracellular ATP on the ciliary beat, as a P_2 purinoceptor. On the other hand, p96 may represent a P_2 purinoceptor or an ectonucleotidase.

Key words: Purinoceptors — Membrane receptors — Affinity labeling — Ciliary activity

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Introduction

Cilia are cellular protrusions covered by cellular membrane which play a predominant role in cell motion through their periodic and synchronous beating. They exist in a wide range of organisms and tissues from protozoa to the digestive, reproductive and respiratory systems of vertebrates in which they transport mucus, a viscoelastic layer of glycoproteins covering the cilia. Normal mucociliary activity is essential for the regular function of the systems in which the cilia are found.

Ciliary cells are excitable in the sense that they change their activity dramatically in response to various stimuli, among which extracellular ATP is prominent [44, 45, 50, 40]. ATP in micromolar concentrations enhances the ciliary activity, by 3–4-fold in frequency and 4–5-fold in the rate of the transport [33, 46, 19], and stimulates mucin release [25]. The stimulation is transient, indicating that multiple steps involving ATP binding may take place in these processes.

The molecular events underlying the stimulation of ciliary activity by extracellular ATP were examined recently [46]. Ca^{2+} or Mg^{2+} were found to be essential for this effect, and the hydrolysis of ATP was not required. Also, the activation of Ca^{2+}-dependent K^+ channels, but not voltage-gated Ca^{2+} channels seems to play a direct role in the effect of ATP on the ciliary activity. In view of these findings, the following model has been proposed: in the presence of Ca^{2+} or Mg^{2+}, extracellular ATP binds to a membrane receptor and triggers Ca^{2+} mobilization from intracellular stores. Consequently, Ca^{2+}-dependent K^+ channels are activated, leading to K^+ efflux and membrane hyperpolarization. The intracellular Ca^{2+} elevation and membrane hyperpolarization lead to the enhancement of the ciliary activity [46]. This model implies the existence of specific receptor(s) for

ATP at the extracellular membrane surface of ciliated cells, and points towards a possible role for ATP as a local regulator in this system.

In the last decade there has been a growing recognition of the role of extracellular ATP as a neurotransmitter (or local mediator) [3, 22, 16, 10, 8], distinct from its standard functions as an intracellular source of chemical energy. Very little is known about the molecular properties of the receptors by which the effects of ATP are exerted. Nevertheless, they were characterized pharmacologically as P_2 purinoceptors, as opposed to the P_1 or A receptors for adenosine [3, 4]. They have been further subclassified into P_{2x}, P_{2y} [5], P_{2z} and P_{2t} [22] and more recently P_{2u} [32, 6], which were identified in various systems [31, 43]. Due to a lack of specific inhibitors, the discrimination between the classes lies on the rank order of potency of ATP and its structural analogues and possibly on the requirement to divalent cations.

Although the physiological importance of P_2 receptors is widely accepted, more experimental work is needed to identify and characterize these receptors at the molecular level. The ATP analogue BzATP[1] has been successfully used as a photoaffinity label for ATPases [48] and other ATP binding proteins [29, 51]. Using ^{32}P-labeled BzATP, a 53 kDa membrane protein from turkey erythrocyte membranes was labeled, which is likely to represent a P_{2y} receptor [1]. BzATP has also been shown to be a potent agonist to ATP in various P_2 purinoceptors-activated systems [2, 21, 12, 11, 31, 30]. Recently, a P_{2u} receptor from human airways was cloned and expressed [34].

In the present work we used [α-^{32}P]BzATP to label, identify and further characterize the ATP receptor(s) in ciliated cells from frog esophagus. [α-^{32}P]BzATP specifically labeled two proteins: p46 and p96. The effect of various nucleotides on this photoaffinity labeling was compared to that on the enhancement of ciliary beat frequency in cell culture. The results are discussed in terms of the involvement of these proteins as nucleotide receptors or nucleotidases in transducing the ATP signal to control the ciliary activity.

Materials and Methods

Nucleotides, EGTA, Hepes, trypsin (type III), soybean trypsin inhibitor (type II-S) were obtained from Sigma; EDTA was from Aldrich, and [α-^{32}P]ATP was from Amersham. Ca/Mg-free PBS and antibiotic mix solution were purchased from Bio-Lab, Jerusalem. Foetal Calf Serum and L-15 Leibovitz medium were from Biological Industries, Beit-Haemek. BzATP and [α-^{32}P]BzATP were synthesized and purified as described by Williams et al. [49].

Biological Material

The experiments were carried out on minced tissue pieces or monolayer tissue culture of frog esophagus, using the procedure described previously [14]. Briefly, frogs (*Rana ridibunda*) were killed by pithing the brain and spinal cord; the esophagus was removed and washed three times in sterile Ca/Mg-free PBS. After most of the mucus and the blood was removed, the esophagus was minced to produce small (3 × 3 mm) pieces of tissue in a clean environment. The tissue pieces were kept on ice-cold PBS until use.

Alternatively, the tissue was washed in sterile PBS containing antibiotics (100,000 U penicillin, 100 mg streptomycin and 10,000 U mycostatin/100 ml). The esophagus was minced in culture medium (15% Foetal Calf Serum, 65% L-15 Leibovitz medium, 20% sterile distilled water, supplemented with antibiotics at a 1:10 dilution). 2–4 tissue pieces were placed on plastic Petri dish (35 mm, Nunc) and overlaid with 0.5–0.7 ml of culture medium. The explants were left undisturbed for two hours. The culture medium was changed every two days and 5–21-days-old tissue cultures were either used for frequency measurements on monolayers, or harvested.

Cell suspensions were prepared from tissue cultures containing more than 80% active ciliated cells (as visualized by microscope). The solution used for harvesting was a modified Ringer's solution with increased buffer capacity. It contains (in mM): NaCl, 90; KCl, 2.5; Na-Hepes, 30, pH 7.2; and Na-P_i, 1.9. When required, EGTA, $CaCl_2$, $MgCl_2$ or other compounds were added to the harvesting solution. The cultures were washed three times in harvesting solution, and 900 μl of this solution supplemented with 5 mM EGTA were added. Cells were mechanically separated by pumping the solution in and out a micropipette for 1 min. The original tissue was then discarded and mechanical separation continued for 4 additional minutes. The separation was stopped by addition of 100 μl of harvesting solution containing 45 mM $CaCl_2$. The cells were collected by centrifugation at 250 × *g* for 10 min, washed in Ca/Mg-free harvesting solution and resuspended in minimal volume.

Measurements of the Ciliary Beat Frequency

Ciliary beat frequency was measured using the dual computerized photoelectric device previously described [15]. This method is based on measurement of scattered light from two small ciliary areas (2.5 μm in diameter) under the microscope with an objective magnification of ×20. The light is collected by two optical fibers (50 μm in diameter) placed in the focal plane of the ocular, and detected by two photomultipliers. The photoelectric signals are further amplified, digitized and collected at a sampling rate of 360 Hz. The ciliary beat frequency was derived from the power spectrum obtained by Fast Fourier Transform of the data [15]. The frequency, among other wave characteristics [36, 17, 18], represents the quantitative parameter of choice to indicate the transition of a ciliary system from "normal" to "stimulated" activity.

Ciliary frequency was measured in Ringer's solution containing (in mM): NaCl, 120; KCl, 2.5; $CaCl_2$, 1.0; and Na-Pi, 1.9, pH 7.2. The tissue culture was washed three times and left undisturbed for 20–30 min in 900 μl of this solution prior to the measurements. Cells displaying steady ciliary activity with a beat frequency in the range 7–11 Hz were used for stimulation experiments. The reference fre-

[1] The abbreviations used are: ADPβS, adenosine 5′-O-(2-thiodiphosphate); AMP-PCP, β,γ-methylene-adenosine 5′-triphosphate; AMP-PNP, β,γ-imido-adenosine 5′-triphosphate; BzATP, 3′-O-(4-benzoyl)benzoyl ATP; 2MeSATP, 2-methylthio ATP; G-protein, GTP binding regulatory protein; GTPγS, guanosine 5′-O-(3-thiotriphosphate); PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; UV, ultraviolet

quency (F_o) was the average of three consecutive samplings of 40 sec each, separated by 200-sec intervals. Then, without moving the tissue, 100 µl of Ringer's solution containing ATP or one of its analogues was added to reach a final concentration in the range of 10^{-8}–10^{-4} M. The activity was monitored for 10 min, immediately after the addition. The frequency was determined as an average over 40 seconds and normalized to F_o. Each experiment was repeated with different Petri plates. In some experiments, the cell cultures were irradiated with UV light (*see* conditions below) for 1 min in the presence or absence of nucleotides. Some protocols also included a wash step in Ringer's solution designed to remove the added nucleotides.

Photoaffinity Labeling of Intact Cells by [^{32}P]BzATP

Unless otherwise specified, cells in suspension (0.5–3 µg protein) or intact pieces of esophagus were preincubated for 5 min in the dark with 1 µM [α-^{32}P]BzATP (specific activity 2–4 10^6 cpm/nmol) in 35 µl of harvesting solution supplemented with 1.0 mM $CaCl_2$. The samples were irradiated for 3 min using a 15W UV lamp (Tana, Israel) at a distance of 5 cm. The reaction was stopped in the dark by adding 4–fold concentrated sample buffer containing: 250 mM Tris-Cl, pH 6.8, 40% (v/v) glycerol, 8% (w/v) SDS, 4% (v/v) β-mercaptoethanol, and incubation for 3 min at 100°C. The samples were analyzed by SDS-PAGE as described below. Control experiments indicate that under the conditions we used (i) the light absorption by nucleotides is negligible (1–2 mm width of 0.1 mM solution above the sample); (ii) no light-dependent labeling by [α-^{32}P]ATP is detectable; and (iii) no change in the SDS-PAGE protein profile is apparent following irradiation.

Gel Electrophoresis

The analysis of protein profile by SDS-polyacrylamide slab gel electrophoresis was performed using the discontinuous buffer system of Laemmli [26] in 1.5 mm thick slab gels with 4–13% or 10% acrylamide and a 3% stacking gel. The gels were stained with Coomassie Brilliant blue. Autoradiography of the dried gels was carried out using Kodak X-Omat film. Quantitative analysis of the labeled protein bands was determined after proper calibration by densitometric scanning of the autoradiogram, using a densitometer (Molecular Dynamics).

Results

The ability of BzATP without photoactivation, to act as an ATP analogue and elicit a ciliary response is shown in Fig. 1. Both ATP and BzATP cause a dramatic and immediate rise in the ciliary beat frequency followed by a gradual decay. Dose-response curves for the effect of BzATP on ciliary stimulation are parallel to these for ATP (*see* Fig. 8). The maximal enhancement (F_{max}/F_o) caused by BzATP is lower than that observed with ATP (Figs. 1 and 8), which is in agreement with the results observed in other systems [12, 38, 31, 30]. Thus extracellular BzATP stimulates ciliary beat frequency in a manner similar to that of ATP in terms of both the instantaneous enhancement and its decay. Therefore, ^{32}P-labeled BzATP can be used for photoaffinity labeling of the ATP receptor(s) in ciliated cells from frog esophagus epithelium.

The effect of covalent binding of photoactivated

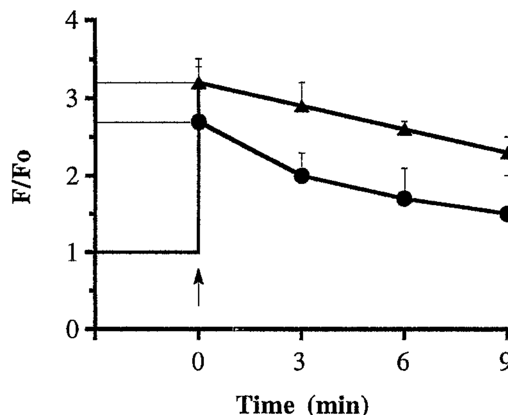

Fig. 1. Time course of the enhancement of the ciliary frequency by ATP and BzATP. Monolayers of ciliated cells were preincubated in Ringer's solution and the basal frequency (F_o) was measured as described under Materials and Methods. A final concentration of 10 µM ATP (▲) or BzATP (●) was added (indicated by the arrow) at time zero, and the beat frequency was measured at the times indicated in the abscissa. At each time point, F represents the average frequency over 40 sec. The averages and the standard errors for the relative enhancement (F/F_o) for 4 measurements in each case, are plotted *vs.* time. The thin lines indicate the maximal relative enhancement (F_{max}/F_o) obtained with each stimulant.

BzATP on the ciliary response can be demonstrated best following removal of free nucleotides from a stimulated culture. Fig. 2 shows that with nonirradiated cells stimulated by either ATP or BzATP, washing out the nucleotides from the medium results in a complete decay of the beat frequency to the basal level. A similar result was observed with cells irradiated with UV light in the presence of ATP. However, in the presence of BzATP during irradiation, the stimulatory effect is sustained and resistant to washing. Control experiments indicate that under the conditions we used, no damage to the basal ciliary activity is detected following irradiation; however, the UV light treatment seems to impair slightly the stimulation by ATP. These results nevertheless support the view that covalent binding of BzATP to a cellular component results in a persistent stimulation of the ciliary activity.

In view of the large ATP binding capacity inside cells, the identification of extracellular ATP receptors requires cellular preparations free from accessible intracellular proteins. In our labeling experiments intact ciliary cells from frog esophagus were either in their native environment as washed tissue pieces, or isolated as a suspension from tissue culture monolayers as described in Experimental Procedures. Both preparations were irradiated with UV light in the presence of [α-^{32}P]BzATP. The protein profiles revealed by SDS-PAGE and Coomassie blue staining are shown in Fig. 3*A*, and the corresponding autoradiograms are shown in Fig. 3*B*. With both cellular preparations, two polypeptides with respective apparent molecular masses of 46 and 96

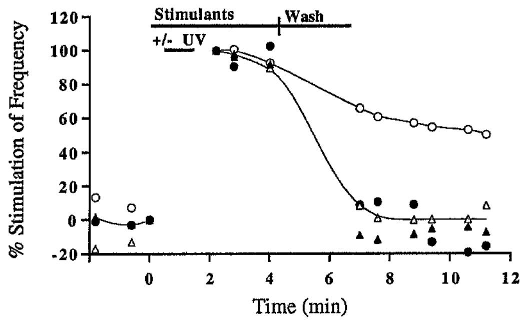

Fig. 2. Effect of UV irradiation and washing on the relative enhancement of ciliary beat frequency by ATP or BzATP. The experiment was conducted with 4 separate Petri plates as described in Fig. 1, except that each data point represents an average over 30 sec. The basal frequency was measured over 2 min before the addition of 10 μM ATP (▲, △) or BzATP (●, ○) at time zero. The last of these measurements was defined as F_o. The monolayers were incubated with the stimulants either in the dark (filled symbols) or irradiated with UV light for 1 min (open symbols), followed by a dark period during which ciliary beat frequency was measured. The first of these measurements was considered as the maximal frequency $\tilde{F}_{max}$, by analogy to Fig. 1. Four minutes after the addition of nucleotides, the medium containing the stimulants was washed out and replaced by fresh Ringer's solution, and additional measurements were made. The illumination and washing regime is shown at the top of the figure. The data were processed and the relative stimulation in each experiment was calculated as $100 \cdot (F - F_o)/(\tilde{F}_{max} - F_o)$, where F stands for the frequency measured at the indicated time. The values for maximal stimulation ($\tilde{F}_{max}/F_o$) are: ATP-UV (▲), 2.3; ATP + UV (△), 1.7; BzATP-UV (●), 1.6; BzATP + UV (○), 1.7.

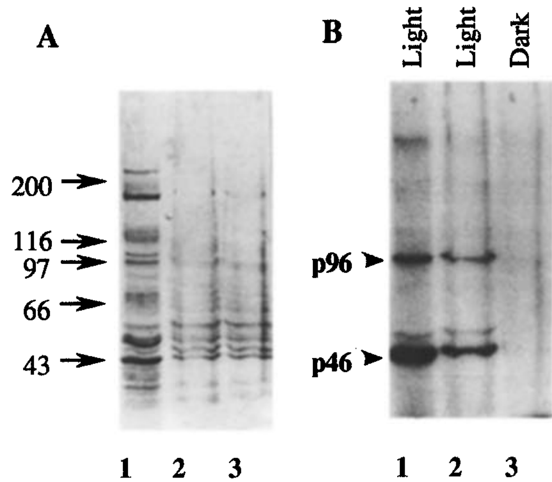

Fig. 3. [α-^{32}P]BzATP labeling of intact cell proteins from native and cultured frog esophagus. A small piece of frog esophagus (lane 1), and an aliquot of cultured cells in suspension (lanes 2 and 3) were labeled with [α-^{32}P]BzATP as described under Materials and Methods. The proteins were separated by SDS-PAGE (4–13% polyacrylamide). The gel stained with Coomassie brilliant blue is shown in panel *A*, and the corresponding autoradiogram in panel *B*. The positions and relative Mr (×10^{-3}) of molecular weight standards (Bio Rad) are indicated in panel *A*. The apparent Mr of the prevalent labeled proteins (p46 and p96) shown in panel *B* was assessed from a calibration curve.

kDa, designated thereafter as p46 and p96, were labeled with ^{32}P. Although the tissue pieces yield a protein profile different than that of the cell suspension (compare lanes 1 and 2 in Fig. 3*A*), the labeling pattern by [α-^{32}P]BzATP is very similar in both preparations. Covalent labeling of both proteins by BzATP strictly requires irradiation. Similar experiments performed with frog palate epithelium (tissue pieces and cells in culture) yielded a similar labeling profile (*data not shown*). The apparent Mr values for p46 and p96 were not altered when the duration of UV irradiation was varied (*not shown*). The intensity of label in p46 is higher (2- to 4-fold) than that in p96.

The dependence of photoaffinity labeling of proteins in cell suspensions on ligand concentration is presented in Fig. 4. The incorporation of label from [α-^{32}P]BzATP in both p46 and p96 reaches a maximal value at rather low concentrations, with apparent half maximal binding at about 40 nM, which promotes only a poor stimulation (1.1–1.3-fold) of ciliary activity without photoirradiation. This value reflects the irreversible covalent binding step and by no means represents the true dissociation constant at equilibrium which must be lower. On the other hand, photolabeling follows pseudo-first order kinetics ($t_{1/2} = 0.5$ min), so that the incorporation of BzATP is completed in about 3 min of UV irradiation (*not shown*). Thus, under the conditions we used for photo-

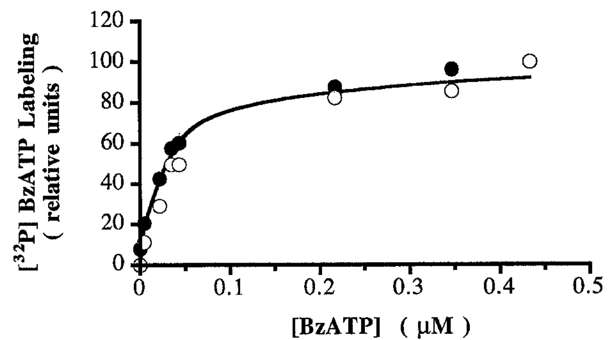

Fig. 4. Photoaffinity labeling of cultured cells as a function of BzATP concentration. Cells in suspension were labeled with the indicated [α-^{32}P]BzATP concentrations and subjected to SDS-PAGE (10%), autoradiography and to quantitative analysis as described under Materials and Methods. The label found in p46 (○) or in p96 (●) were normalized to the maximal value obtained for each polypeptide. In this experiment the ratio between the label found in p46 and p96 was 2.2.

affinity labeling ([BzATP] ⩾ 0.2 μ*M*), the binding sites should be fully occupied. Assuming a binding stoichiometry of 1 for each protein, quantitative analysis of the data in Fig. 4 yields maximal covalent binding capacities of 85 and 18 pmol/mg cell protein for p46 and p96, respectively.

In some preparations, an additional polypeptide band of Mr = 55,000 was labeled upon irradiation. The appearance of this labeled band (comigrating with tubulin, *not shown*) was incidental and probably reflects a

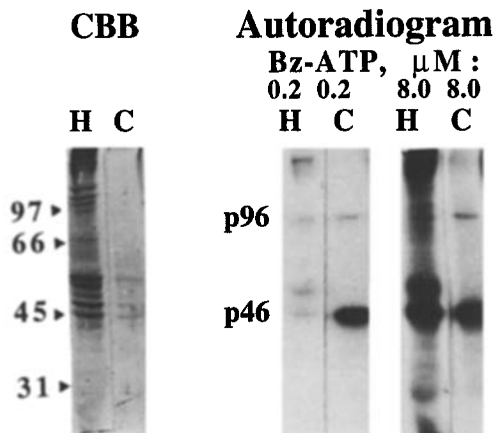

Fig. 5. Photoaffinity labeling of cultured ciliary cells in suspension and esophagus epithelium homogenate at two [α-^{32}P]BzATP concentrations. The epithelium containing mainly ciliary cells was scrapped from the internal surface of frog esophagus, thoroughly washed and homogenized by 20 strokes of a Duall micro tissue grinder in Ca/Mg-free harvesting solution. Cells in suspension (*C*) and homogenate (*H*) aliquots (1.1 and 5.8 μg protein, respectively) were covalently labeled with [α-^{32}P]BzATP at the indicated concentrations and processed as described in Materials and Methods. The left panel represents Coomassie brilliant blue stain of the SDS-PAGE lanes and the corresponding autoradiograms are brought in the right panel.

minor population of permeabilized cells. In order to critically assess the possible contribution of intracellular binding sites, we compared photoaffinity labeling of intact cells in suspension with that of esophagus epithelium homogenate, in which intracellular binding sites are exposed to the medium, by [α-^{32}P]BzATP at two concentrations. The results are presented in Fig. 5. At relatively low BzATP concentration (0.2 μM), p46 and p96 are exclusively labeled in intact cells, while two additional bands (55 and 400 kDa) also appear in the homogenate. The labeling efficiency was much lower in the homogenate in spite of a higher protein content, probably reflecting dilution of the label by released intracellular nucleotides. Increasing the concentration of [α-^{32}P]BzATP by 40-fold affects neither qualitatively nor quantitatively the labeling pattern in intact cells. However, with the homogenate, 8 μM BzATP strongly labeled many bands including new polypeptides, which became apparent. It should be noted that with the homogenate, the label increased in p46 but not in p96, indicating that at high BzATP concentration the former may reflect additional polypeptide(s) with intracellular binding sites. Similar results were observed with cells permeabilized with detergents or alamethicin [39] (*not shown*). These results indicate that in intact cells, p46 and p96 exclusively labeled at low BzATP concentrations, contain ATP binding site(s) accessible from the extracellular fluid.

Consequently, we expect that the nucleotide binding activity must be susceptible to exogenous proteases added to the bathing solution. Intact cells in suspension

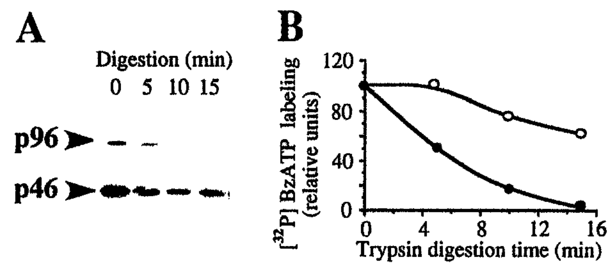

Fig. 6. [α-^{32}P]BzATP photoaffinity of trypsin-treated cells. Trypsin was added to a final concentration of 2.5 μg/ml to a cell suspension (62.5 μg/ml) in Ca/Mg-free harvesting solution. At the indicated time, the digestion was stopped by adding a 20-fold molar excess of soybean trypsin inhibitor. The resulting mixtures were further subjected to photoaffinity labeling by [α-^{32}P]BzATP, SDS-PAGE (10%) and autoradiography as described in Materials and Methods. *A:* autoradiogram; *B:* quantitative analysis of the autoradiogram shown in panel *A*. The relative labeling in p46 (○) and p96 (●) was quantitated by densitometric scanning of the autoradiogram. The ratio between the label found in p46 and p96 at zero time was 2.9 in this experiment.

were subjected to limited proteolysis by trypsin. The results of [α-^{32}P]BzATP labeling of trypsin-treated cells are shown in Fig. 6. Although p96 is more sensitive, the labeling of both proteins decreased upon digestion with trypsin under mild conditions. This demonstrates that the BzATP binding sites in both proteins are at least partially exposed to the medium (i.e., located on the extracellular face of the membrane).

The dependence of labeling of p96 and p46 by [α-^{32}P]BzATP on divalent cations is shown in Fig. 7 and Table 1. When EDTA was added to cells previously washed in a Ca/Mg-free suspension buffer, the labeling of p96 slightly increased. In contrast, the labeling of p46 was almost completely inhibited in the presence of EDTA (Fig. 7). As shown, the labeling of p96 by [α-^{32}P]BzATP increases by about 30%, while that of p46 is decreased by about 70% upon addition of 0.5 mM EDTA to Ca/Mg-free cells suspension. Table 1 summarizes the results from six experiments similar to that shown in Fig. 7. Control experiments indicate that a substantial labeling (*ca.* 80%) of p46 by [α-^{32}P]BzATP is still obtained with Ca^{2+} concentrations as low as 10 μM (*data not shown*). These results indicate that the labeling of p46 but not p96 by [α-^{32}P]BzATP strictly requires Ca^{2+} or Mg^{2+}.

We have previously shown [46] that ATP analogues can serve as agonists in the stimulation of ciliary activity. If ATP binding to a receptor is a prerequisite for the stimulation of ciliary beat frequency, we expect the potency of ATP analogues in stimulation to be correlated to some extent with their capacity to block photoaffinity labeling of the ATP receptor by BzATP. We examined the effect of several nucleotides on the covalent labeling of p46 and p96 by [α-^{32}P]BzATP, along with their effect on ciliary beat frequency (Figs. 8, 9, and Table 2).

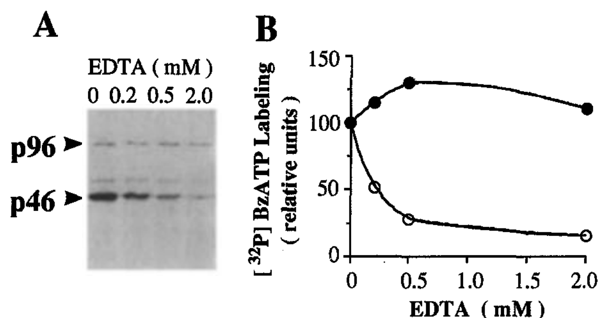

Fig. 7. The effect of EDTA on the [α-^{32}P]BzATP labeling of p46 and p96. EDTA, at the indicated concentrations, was added to cell suspensions in Ca-Mg-free harvesting solution and labeled with [α-^{32}P]BzATP as described under Materials and Methods. *A:* autoradiogram; *B:* quantitative analysis of the autoradiogram shown in panel *A*. In panel *B*, the relative labeling in p46 (○) and p96 (●) is plotted as a function of EDTA concentration.

Table 1. The divalent cation requirement for the [α-^{32}P]BzATP labeling of p46 and p96

Treatment	[α-^{32}P]BzATP labeling (% of control)	
	p46	p96
EDTA 0.5 mM + Ca^{2+} 1.5 mM	93 ± 8	80 ± 10
EDTA 0.5 mM + Mg^{2+} 1.5 mM	105 ± 11	110 ± 15
EDTA 0.5 mM	35 ± 17	120 ± 20

Aliquots of cell suspension in harvesting solution containing EDTA (0.5 mM) and as indicated Ca^{2+} or Mg^{2+} (1.5 mM), were incubated with 1 μM [α-^{32}P]BzATP, irradiated and processed for SDS-PAGE and label quantitation as described under Materials and Methods. The label incorporated in p46 and p96 was normalized to the value obtained with suspensions containing 1.0 mM Ca^{2+} only in the photolabeling reaction mixture. The results represent the averages and standard deviations of 6 different experiments.

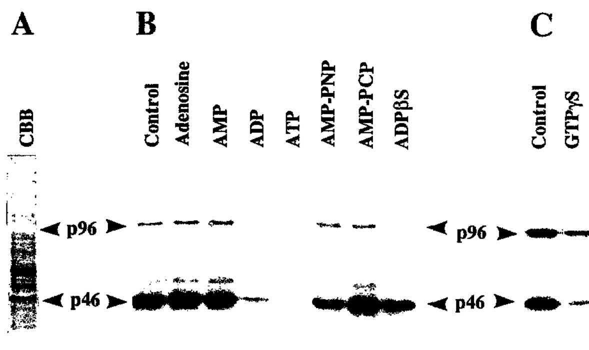

Fig. 8. Effect of nucleotides on the photoaffinity labeling of p46 and p96 by [α-^{32}P]BzATP in cells suspension. Intact cells were preincubated and irradiated as described under Materials and Methods in the presence of 1 μM [α-^{32}P]BzATP only (Control), or together with 0.1 mM of the indicated compounds. The control experiment contained [α-^{32}P]BzATP only. The proteins were separated by SDS-PAGE (10%). Panel *A:* Coomassie brilliant blue (CBB) stain of one representative lane. Panels *B* and *C:* Representative autoradiograms obtained with different cell cultures.

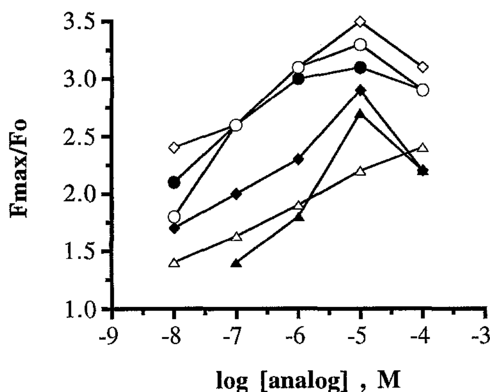

Fig. 9. Effect of nucleotides on the ciliary beat frequency of cultured frog esophagus. The ciliary beat frequency was measured following the addition of UTP (◇), 2MeSATP (○), ATP (●), ADP (♦), BzATP (▲) and AMP-PCP (△), as described in Materials and Methods. The maximal frequency enhancement after the addition of stimulant is represented by the ratio F_{max}/F_o, as shown in Fig. 1. Each point represents the average of 10–15 different experiments. The standard deviations are in the range of 0.4–0.6.

Tissue pieces or cell suspensions were irradiated with [α-^{32}P]BzATP in the presence of ATP analogues, and the proteins were separated by gel electrophoresis. The experimental conditions were such that the photolabel was saturating (1 μM, *see* Fig. 4), and the challenging analogues were added in excess. Figure 8 shows representative results of the effect of several analogues on the [α-^{32}P]BzATP labeling of p46 and p96 in cell suspensions. The extent of labeling of p46 and p96 was quantitatively analyzed by densitometric scanning and the average values are presented in Table 2. All compounds tested decreased the incorporation of label in both polypeptides to variable extent (Fig. 8 and Table 2). The rank order of efficiencies for inhibition of p46 labeling is: ATP ≅ ADP > GTPγS > ADPβS, UTP, 2MeSATP, AMP-PNP > AMP-PCP > AMP > adenosine, while for p96 is: ADP ≅ ADPβS ⩾ ATP ⩾ AMP-PCP, AMP-PNP > GTPγS ⩾ AMP > 2MeSATP, UTP, adenosine. The same rank order of efficiencies was obtained with 2-fold lower concentrations of the analogues and with esophagus tissue pieces (*data not shown*).

Table 2 also summarizes the capacity of the analogues to stimulate the ciliary beat frequency in tissue culture. With most nucleotides tested, the dose response is biphasic, as shown in Fig. 9. At low concentrations, an increase in ciliary beat is observed, while this effect is reversed at high concentrations. As suggested by the monophasic increase observed with the nonhydrolyzable AMP-PCP, the biphasicity may reflect secondary effects through binding of the nucleotides or their degradation

Table 2. The effect of nucleotides on ciliary beat frequency and on [α-^{32}P]BzATP labeling of p46 and p96

Compound	Ciliary beat stimulation (F_{max}/F_o)	Inhibition of BzATP labeling (% reduction in band intensity)	
		p46	p96
AMP	1.2 ± 0.3	17 ± 10	10 ± 10
Adenosine	1.7 ± 0.4	6 ± 6	6 ± 6
AMP-PCP	2.2 ± 0.4	33 ± 12	32 ± 7
ADP	2.8 ± 0.4	93 ± 3	92 ± 1
ATP	3.1 ± 0.6	96 ± 2	84 ± 6
AMP-PNP	3.2 ± 0.6	58 ± 10	28 ± 5
2MeSATP	3.3 ± 0.4	60 ± 5	6 ± 5
ADPβS	3.6 ± 0.5	63 ± 15	90 ± 2
UTP	3.5 ± 0.6	63 ± 8	6 ± 5
GTPγS	3.2 ± 0.4	73 ± 2	13 ± 10

The stimulation of ciliary beat frequency elicited by 10 μM of the indicated compound was assessed as described under Materials and Methods. F_{max}/F_o represents the maximal relative enhancement as described in Fig. 1. The relative labeling from 1 μM [α-^{32}P]BzATP in the presence of 0.1 mM analogue was determined by densitometric scanning of autoradiograms, and expressed relative to control experiments run with no added competing nucleotide (*see* Fig. 9). The values shown are averages and standard deviations of 8–10 different experiments in each case. The ciliary beat stimulation values measured for the following pairs: Adenosine/AMP; ATP/AMP-PCP and AMP/AMP-PCP were submitted to statistical analysis. By student's *t*-test analysis the differences were found to be significant ($P < 0.01$; 0.002 and 0.0002, respectively).

products to additional sites. We used a concentration of 10 μM at which an optimal stimulation was observed for nucleosides phosphate in the range 10^{-8}–10^{-4} M. The rank of agonist potency for stimulation of ciliary activity is: ADPβS, UTP ⩾ 2MeSATP, GTPγS, AMP-PNP, ATP ⩾ ADP > AMP-PCP > adenosine > AMP (Table 2). GTPγS is commonly used for testing the involvement of the GTP-binding proteins [20]. However, since it is applied to intact cells, and it does stimulate the ciliary beat frequency in vitro as well as ATP (Table 2), it should act here as an extracellular stimulant and bind to extracellular sites.

The results in Table 2 indicate that ATP, ADP, AMP-PCP, AMP and adenosine induce comparable effects on ciliary beat stimulation and prevention of p46 and p96 labeling by [α-^{32}P]BzATP. The higher apparent affinity of ATP and ADP for the binding sites in both proteins is also reflected in the relative potency of these nucleotides to increase the ciliary beat frequency. AMP-PCP inhibits the labeling of both proteins by about 30%, which is consistent with its relatively weak stimulation of ciliary activity. The minor effects of AMP and adenosine on the ciliary beat frequency are also well correlated with their effect on labeling. On the other hand, the effects of UTP, 2MeSATP, GTPγS, ADPβS and AMP-PNP are more complex. They increase the beat frequency to the same extent or more as compared to ATP, but they prevent only partially the incorporation of label in both p46 and p96.

Discussion

Although stimulation of ciliary activity by extracellular ATP has been recognized for a long time, little work was done to investigate the ATP receptors in these systems. Also, the pharmacological characterization in terms of the rank of potency of nucleotides to mimic the ATP effect in vitro was not studied. The present work using the photoaffinity probe BzATP is the first report on possible identification of proteins with extracellular ATP binding sites involved in ciliary activation.

Like extracellular ATP, BzATP stimulates the ciliary activity with similar dose response (Fig. 9) and time course (Figs. 1, 2). This functional similarity justifies the use of ^{32}P-labeled BzATP for the identification and characterization of potential ATP receptor(s) in epithelial cells from frog esophagus. As suggested by the biphasic effect on the ciliary beat frequency time course, the dose response indicates that the nucleotides (or degradation products) may act at more than one site with different affinities. Irradiation of intact cells with ultraviolet light in the presence of [α-^{32}P]BzATP, and subsequent analysis of the proteins revealed the photoaffinity labeling of mainly two polypeptides, p46 and p96. One or both proteins must be involved in the mechanism of stimulation of ciliary activity by ATP. Since an extracellular signal is transduced into a cellular function, it is important to address the question of the labeled proteins location.

Cellular Location of p46 and p96

The use of intact cells insures that the membrane-impermeable [α-^{32}P]BzATP would not label intracellular ATP binding sites [9]. Covalent labeling of both proteins occurs at relatively low concentrations of BzATP (Fig. 4). Such effective range (up to 100 nM) is not expected for cytosolic enzymes which are normally exposed to much higher ATP concentrations. Intracellular location is further ruled out by the following observations. (i) The capacity for label incorporation into p46 and p96 in cell suspensions was retained following pretreatment with EGTA and extensive washing, indicating that they are tightly associated with the cell membrane. (ii) The labeling of both polypeptides by [α-^{32}P]BzATP is susceptible to mild proteolysis (Fig. 6). (iii) The covalent labeling profile of small pieces of esophagus (or palate tissue) was identical to that of cultured ciliary cells in suspension (Fig. 3). (iv) In contrast, with homogenized cells lower levels of labeling is observed at low BzATP concentrations, and two additional different

polypeptides are labeled (Fig. 5). (v) In the homogenate at high BzATP concentrations, while the label in p96 remains constant, a strong increase in p46, as well as in many new proteins, is apparent. The increase in labeled p46, observed also in permeabilized cells, indicate that internal proteins of similar Mr become exposed. This may suggest the existence of a large intracellular pool of receptor sequestered by endocytotic recycling mechanisms [41]. Thus, p46 and p96 are likely to reflect integral membrane proteins which contain ATP binding sites exposed to the medium. Such topology implies a possible role for each labeled protein as either an ATP receptor or an extracellular ATP-metabolizing enzyme.

The Nature of the p46 and p96 ATP Binding Proteins

The combination of an ATP-induced biological effect and the covalent labeling by extracellular [α-^{32}P]BzATP of membrane proteins, points towards the involvement of purinoceptors [10]. These G-protein-linked intrinsic membrane proteins contain seven transmembrane helices, and present a high homology to the transmembrane and G-protein-binding domains of rhodopsin [23].

The label in BzATP was incorporated into two membrane proteins of apparent molecular masses of 46 kDa and 96 kDa. The maximal covalent binding capacity of p46 and p96 in intact ciliary cells in suspension is in the range 20–100 pmol/mg protein, the former bearing more label. This represents a rather high density as compared to that reported for extracellular purinoceptors in other systems (ranging from fmol to few pmol/mg). While the monomeric nature of each labeled protein remains to be established, relatively small sizes are consistent with those of well defined G-protein-linked superfamily receptors. [α-^{32}P]BzATP photolabeled a 53 kDa plasma membrane protein suggested to represent a P_{2y} purinoceptor [1]; and in catecholamine-secreting PC12-cells [38], it labeled two major proteins (44 and 50 kDa) and a minor protein (97 kDa). The 50 kDa labeled protein was suggested to represent the P_2 purinoceptor responsible for ATP-stimulated secretion in these cells. In human leukemia cell line [13], [α-^{32}P]BzATP photolabeled a 53 kDa expressed plasma membrane protein representing a P_{2u} purinergic receptor. Thus, the size of p46 rather than of p96 may point towards its possible identity as a purinoceptor.

The rank order of potency of the nucleotide analogues to mimic ATP provides a tool to distinguish further between the established classes and subclasses of purinoceptors. The small effects observed with AMP and adenosine in protecting p46 and p96 from photoaffinity labeling by [α-^{32}P]BzATP (Fig. 8 and Table 2), rule out the possibility that they belong to the P_1 (or A) class [4], and thus point towards a P_2 class. Since AMP-PCP protects the photoaffinity labeling of both proteins to a much smaller extent than ATP, it is unlikely that they represent P_{2x} receptors for which AMP-PCP represents a potent agonist [5]. The similarity in the effects of ATP and ADP at the concentrations we used does not support the involvement of either P_{2t} or P_{2z} receptors [22]. The possibility that p46 and/or p96 act as receptors of P_{2y} or P_{2u} subtypes must therefore be considered further.

An alternative possible role for extracellular ATP binding proteins is their function as ectonucleotidases. Such activity is often invoked in relation to systems which secrete ATP or are stimulated by extracellular ATP [22]. In the last decade there has been an increasing recognition of their involvement in the control systems modulated by extracellular ATP [35, 9, 37, 10, 8].

In the frog ciliary system, a gradual decay of the frequency is observed following stimulation by extracellular ATP (Fig. 1). The mechanism of this decay is still unclear. Since a similar decay was also apparent when AMP-PNP was used instead of ATP [46], it seems unlikely that ecto-ATPases are directly involved in this decay. However, the hydrolysis of the α-β bond in ATP by specific ectonucleotidases [22, 24] cannot be ruled out as a possible desensitization mechanism. As a matter of fact, the use of [α-^{32}P]BzATP as the photoaffinity ligand for nucleotide binding sites may in principle result in the detection of label in either BzAMP, BzADP or BzATP covalently bound to proteins.

Preliminary experiments conducted with tissue pieces, cultured cells in suspension or isolated cilia indicate the presence of a Ca/Mg-dependent ecto-ATPase activity (*unpublished results*). The existence of a homodimeric 5′-nucleotidase (monomer mass 60–80 kDa) located at the extracellular face of the plasma membrane has been reported in many systems [52]. Moreover, a 105 kDa protein, identified as an ecto-ATPase, has been cloned [28] from rat hepatocytes and was found to be identical to a cell-adhesion molecule [27]. Both ectonucleotidases are anchored to the membrane by a single transmembrane segment. The higher sensitivity of p96 labeling to proteolysis (Fig. 6) may suggest that the protein is relatively loosely associated with the membrane. Thus, it is possible that p96 represents an ecto-enzyme. We found that p46—but not p96—requires a divalent cation for its labeling by [α-^{32}P]BzATP (Fig. 7 and Table 1). The bidentate cation-ATP complex [Ca/Mg-ATP]$^{2-}$ is usually the substrate for nucleotidases. While the absence of divalent cation should impair the activity of nucleotidases, the effect on ATP *binding* remains uncertain, since free ATP^{4-} often represents a potent competitive inhibitor. The results of labeling alone are insufficient to ascertain a role for p96 as nucleotidases in our system, and different approaches, involving the identification of bound and free products resulting from catalysis, should be used critically to address this question.

THE RELATION BETWEEN THE LABELING OF p46 AND p96 AND THE STIMULATION OF CILIARY ACTIVITY BY ATP

The ciliary beat frequency represents the ultimate response of the ciliary cells to stimulus by extracellular ATP. A comparison between the results of frequency measurement and these obtained from the affinity labeling of p46 and p96 by [α-^{32}P]BzATP provides a way to assess the involvement of the labeled proteins in the ciliary stimulation by extracellular ATP. However, we do not necessarily expect a strict correlation between the binding of ATP to a receptor and the biological response.

We recently showed [46] that Ca^{2+} (1.0 mM), which can be replaced by Mg^{2+} (0.1 mM), is essential for frequency stimulation by extracellular ATP. We proposed that extracellular divalent cations either play a regulatory role or are required for ATP binding [46]. The BzATP labeling of the 46 kDa but not of 96 kDa protein is strictly dependent on divalent cations (Fig. 7 and Table 1). However, the concentration required for p46 labeling is substantially lower than that required for the ATP stimulation of ciliary activity, indicating a higher affinity to divalent cations for ATP binding (as is the case of P_{2x}, P_{2z} and P_{2u} receptors). The cations may rather fulfill a regulatory function at a later stage of the sequence leading to ciliary beat stimulation, in addition to their involvement in ATP binding.

Our results indicate that UTP (a P_{2u} agonist), 2MeSATP and ADPβS (P_{2y} agonists), enhance ciliary beat frequency as well as does ATP (Fig. 9 and Table 2). Both P_{2y} and P_{2u} purinoceptors are believed to mediate the ATP stimulatory effects via activation of phospholipase C, followed by IP_3 elevation and mobilization of intracellular Ca^{2+} [32, 7, 47]. Such a mechanism for ciliary stimulation by ATP is consistent with our early proposal [46] which was recently demonstrated [42]. In addition, cooperation between P_{2u} and P_{2y} purinoceptors has been proposed [32, 31]. These considerations may lead to the possibility that in our system two types of purinoceptors are involved. On the other hand, UTP was reported to be as potent as ATP in some cases involving P_{2y} receptors [6]. Moreover, the similar potency of ADP and ATP to stimulate ciliary beat frequency points toward a P_{2y} rather than a P_{2u} subtype [6, 8]. Therefore, it is quite likely that in cultured ciliary cells from frog esophagus extracellular ATP stimulates the ciliary beat frequency via a P_{2y} purinoceptor.

The binding of a stimulant to its site and the physiological effect are located at both ends of a complex sequence of steps which compose the signal transduction cascade. The degree of coupling between these two events may be influenced by various accessory processes within the cascade. Therefore, a full correlation between the apparent affinity of the different nucleotides to the labeled proteins and their ability to stimulate ciliary beat frequency is not to be expected *a priori.* We have found that ADP, AMP-PNP, ADPβS, 2MeSATP, UTP and GTPγS produce an in vitro effect similar to that of ATP. All these nucleotides inhibit the [α-^{32}P]BzATP labeling of p46 by 60% and more (Table 2). If partial saturation of the ATP binding site(s) is sufficient to produce a full effect in vitro, then a good correlation is observed between the rank of apparent affinity of the nucleotides to p46 and their stimulation the ciliary beat frequency. With p96, the correlation between the ability of the various nucleotides to prevent the [α-^{32}P]BzATP labeling and their potency for ciliary beat stimulation is quite weak (Table 2), suggesting a minor role for p96 in the *fast* cellular response. We believe that p46 likely represents a purinoceptor, probably of a P_{2y} subtype, responsible for the ATP effect, while p96 may represent an ectonucleotidase, involved in the modulation of cellular response.

CONCLUDING REMARKS

In epithelial cells from frog esophagus, covalent incorporation of ^{32}P from [α-^{32}P]BzATP revealed the labeling of mainly two proteins: p46 and p96, indicating that they contain extracellular ATP binding site(s). To the best of our knowledge, this is the first labeling of extracellular ATP binding sites in a mucociliary system. We believe that the photoaffinity labeling of the proteins by [α-^{32}P]BzATP, together with their preliminary characterization, demonstrate the existence of at least one type of P_2 receptor in ciliated epithelium from frog oesophagus, the second protein representing probably an ectonucleotidase. The specific labeling of these proteins represents a major step toward the eventual purification and identification of these proteins.

Ciliary systems serve as good models for investigation of signal transduction. The ciliary beat frequency is a sensitive indicator for cellular response to stimuli. Covalent photoaffinity labeling of ATP-binding proteins combined with the assessment of the effect of ATP analogues on the ciliary activity provides a powerful approach to investigate further structure-function relationship in ATP receptors and their involvement in the control of the ciliary activity.

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