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Possible Mechanism of Ciliary Stimulation by Extracellular ATP: Involvement of Calcium-Dependent Potassium Channels and Exogenous Ca^{2+}

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Summary. Ciliary motility was examined optically in tissue cultures from frog palate epithelium and frog's esophagus as a function of extracellular concentration of adenosine 5′-triphosphate (ATP) and related compounds. The addition of micromolar concentration of ATP caused a strong enhancement of frequency and wave velocity in the direction of the effective stroke. Since adenosine 5′-[β,γ imido]-triphosphate (AMP-PNP), a nonhydrolyzable analog of ATP, produces the same effects, ATP hydrolysis is not required. The overall potency is ATP $\cong$ AMP-PNP > ADP $\gg$ adenosine > AMP. It is suggested that both the phosphate and the base moieties are involved in ATP binding.

The enhancement of ciliary activity by extracellular ATP is dependent on the presence of extracellular Ca^{2+}, which can be replaced by extracellular Mg^{2+}. The effect of a number of potent inhibitors of the voltage-gated calcium channels on the stimulation of ciliary activity by ATP were examined. No effect was detected in the concentration range within which these agents are specific. On the other hand, quinidine, a potent inhibitor of K^+ (calcium-dependent) channels, inhibits the effect of ATP.

The following model is suggested: exogenous ATP interacts with a membrane receptor in the presence of Ca^{2+}, a cascade of events occurs which mobilizes intracellular calcium, thereby increasing the cytosolic free Ca^{2+} concentration which consequently opens the calcium-activated K^+ channels, which then leads to a change in membrane potential. The ciliary response to these changes is the enhancement of ciliary activity.

Key Words extracellular ATP · ciliary activity · $K^+(Ca^{2+})$ channels · quinidine · Ca^{2+} channels

Introduction

Cilia are densely packed cellular protrusions, numbering 100–200 per cell. Their main function is transport of water or mucus. In order to fulfill this task, cilia perform synchronous periodic beating and form a wave in space and in time, called the metachronal wave. Cilia are divided into cilia which propel water and cilia which propel mucus (a "coat" of viscoelastic fluid 1–30 μm in thickness which covers the ciliated epithelium). The water-propelling cilia are found in ciliated protozoa and in molluscs, are relatively long (10–30 μm) and are not densely packed. Mucus-transporting cilia occur in respiratory, reproductive and digestive systems of mammals and amphibians. They are short (5–7 μm) and densely packed, and as a result, they are quite difficult to investigate. Ciliary cells are excitable cells in the sense that they can respond to a variety of stimuli, mechanical, electrical, chemical and hormonal, by altering the pattern of the ciliary activity. In water-propelling cilia, a systematic study of the influence of the membrane potential on the ciliary activity was performed (Andrivon, 1988; Machemer, 1990). In addition the direct influence of Ca^{2+} ions on the pattern and the frequency of the ciliary beating of demembranated protozoa was investigated (Naitoh & Kaneko, 1973). It is now accepted that these two factors play an important role in controlling the ciliary activity of water-propelling cilia. With mucus-transporting cilia little work was done mainly because of experimental difficulties. There is evidence that the membrane potential or the intracellular concentration of Ca^{2+} participate in controlling ciliary activity (Eckert, 1972; Eckert & Murakami, 1972; Verdugo, 1979; Sanderson & Dirksen, 1986; Villalon, Hinds & Verdugo, 1989). Questions such as whether the membrane potential and the intracellular calcium ion concentration are coupled or independent and what is their relative importance or whether the extracellular calcium or calcium from intracellular storage is used are still to be answered.

The pronounced effect of extracellular ATP on ciliary transport rate and frequency has been known for a long time (Varhaus & Deyrup, 1953; Usuki, 1959; Murakami, Machemer-Rohnisch & Eckert, 1974; Nelson & Wright, 1974; Ovadyahu, Eshel & Priel, 1988; Villalon et al., 1989). As far as we know, this is the strongest effect produced by any extracellular compound on ciliary beating. The concentration of ATP required to increase the ciliary frequency is in the range of 10 to 100 μM, a feasible

concentration from the physiological aspects. Even though these effects are well established, the molecular events underlying these changes in ciliary beating are not understood. Recently a detailed examination of most of the metachronal wave parameters as a function of extracellular ATP concentration was performed (Ovadyahu et al., 1988). It was found that all the parameters of the metachronal wave are influenced by extracellular ATP. This observation explains reasonably well the earlier findings of the rise in the transport rate of particles on frog's palate epithelium (Varhaus & Deyrup, 1953) in the presence of ATP. Moreover, based on these data it was suggested that extracellular ATP influences the ciliary system through interaction with ion channels of the cell membrane (Ovadyahu et al., 1988). Obviously, such a conjecture needs either to be proven or refuted, and this is one of the goals of the present work.

This work contributes to the growing recognition that extracellular ATP and related nucleotides and nucleosides at low micromolar concentrations have potent action on a variety of cells, which differ in their origin and physiological function (Trams, 1974; Heppel, Weisman & Friedberg, 1985; Gordon, 1986; Quast & Cook, 1989; Gelband et al., 1990; Saano et al., 1990; Silberberg & Breemen, 1990). It was found that extracellular ATP induces responses in cells with excitable membranes (Benham & Tsien, 1987; Krishtal, Marchenko & Obukhov, 1988) and in secretory cells (Gallacher, 1982). Ciliary cells belong to the first category and are located in the neighborhood of the secretory cells, to which their function is coupled. Three modes of ATP action have been proposed: (i) an increase of K^+, or Rb^+ efflux (Burgess, Claret & Jenkinson, 1979; Gallacher, 1982; Gordon, 1986; Quast & Cook, 1989), (ii) an increase of membrane Ca^{2+} permeability (Perdue, 1971; Burgess et al., 1979; Benham & Tsien, 1987; Benham, 1989) or stimulation of calcium mobilization and inositol triphosphate production (Gallacher, 1982; Charest, Blackmore & Exton, 1985; Dubyak, 1986; Horstman, Tennes & Putney, 1986; Gonzalez, Rozengurt & Heppel, 1989; Rice, Corn & Singelton, 1990), and (iii) activation of nonselective cationic channels (Krishtal et al., 1988). According to Burnstock's classification (Burnstock,1976, 1978) the cell membrane receptors responsible for the sensitivity to ATP are P_2-purinoceptors.

The present work was designed to answer whether the rise in ciliary activity caused by extracellularly applied ATP is mediated by the modification of ionic membrane permeability. We also wished to gain some insight into the mechanism by which ATP stimulates ciliary activity.

Materials and Methods

Preparations and Solutions

Experiments were conducted on monolayer tissue cultures grown from excised frog palate and esophagus (*Rana ridibunda*) using the procedure described previously (Eshel, Grossman & Priel, 1985). The Ringer solution used for the experiments contained (in mM): 120 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 1.1 Na_2HPO_4 and 0.85 NaH_2PO_4. All the drugs and reagents were obtained from Sigma, and all the solutions were adjusted to pH 7.2 before use. Solutions with a low concentration of Ca^{2-} and Mg^{2+} were prepared by adding known amounts of Ca^{2+}, Mg^{2+} and EGTA to free Ca^{2+} Ringer solution. The total concentration of EGTA was up to 0.5 mM. The free ion concentration was calculated using the computer program previously described (Jean & Klee, 1986). In solutions containing La^{3+}, due to the interaction of La^{3+} with phosphate ions, the phosphate buffer in the Ringer was replaced with HEPES buffer (5 mM).

Optical Measurements

The method used here is based on the simultaneous measurement of scattered light from two points on the ciliary epithelium of monolayer. The distance between the two points can be varied from zero (overlap) to several hundreds of micrometers in steps of 0.5 μm. As was shown, this method enables us to measure the frequency and wavelength of the metachronal wave (Eshel & Priel, 1987), the wave velocity (Priel, 1987), the wave direction (Gheber & Priel, 1990) and the degree of synchronization between the beating cilia (Gheber & Priel, 1989).

Procedure

In order to measure the influence of a given material on ciliary activity the following procedure was adopted: (i) The experiments were conducted in tissue medium culture or in Ringer solution according to the material examined. (ii) The medium or the Ringer solution over the tissue culture was changed three times before starting the experiment. (iii) Six measurements of 40 sec each were taken from the ciliary area in the culture medium or Ringer solution. These six measurements took 20 min. The average of the 240 1-sec spectra obtained served as reference for a given experiment (F_0). (iv) A solution to be tested in a known volume and concentration was added to a constant volume of medium or Ringer solution. The effect of the final concentration on the ciliary activity was measured over time. The measurements were taken from the same ciliary area as in the reference. (v) The values of the parameters obtained under the influence of a given substance at each given time were normalized to the reference.

At each concentration of one of the materials, 5 to 20 tissue cultures taken from 2 to 3 animals were examined. This provides a reasonable statistical ensemble. The ability to perform the measurement on the same ciliary cell, before and after adding the material, considerably increases the accuracy of the experiment. To eliminate the possibility of mechanical stimulation due to addition of the solution to the medium, control experiments were performed in the same manner by adding an equivalent amount of medium or Ringer solution alone. No changes were detected in ciliary activity in the control which confirms that the effects are due to the influence of the added material. Experiments de-

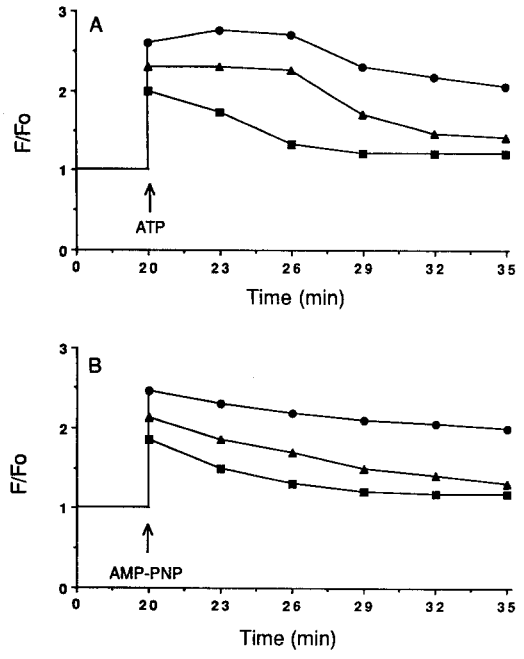

Fig. 1. Typical time courses of the enhancement effect of (*A*) ATP and (*B*) AMP-PNP on the beat frequency of ciliated cells. The basic frequency (F_0) of the ciliated cell was measured in 900 μl of medium solution. After 20 min, 100 μl of ATP or AMP-PNP were added to give the indicated final concentrations of 10 μM (●), 0.1 mM (▲) and 1.0 mM (■). The frequency measurements (F) were continued on the same cell for an additional 15 min. The enhancement effect is represented by the frequency ratio F/F_0.

Table 1. Effect of different compounds on the ciliary frequency and metachronal wave velocity in the direction of the effective stroke

Compound	Concentration (mM)	F_{max}/F_0	V_{max}/V_0
ATP	0.01	2.61	4.0
	0.10	2.17	4.4
	1.00	2.00	2.98
AMP-PNP	0.01	2.45	2.82
	0.10	2.09	3.65
	1.00	1.87	2.24
AMP	0.01	1.00	1.0
	0.10	1.12	1.38
	1.00	1.01	1.41
Adenosine	0.01	1.36	1.34
	0.10	1.8	1.78
	1.00	1.7	1.7
CTP	0.01	1.21	1.45
	0.10	1.83	2.62
	1.00	1.6	1.76
Tripolyphosphate	0.1	1.43	2.26
	0.5	1.54	1.5
	1.0	1.00	1.66
Pyrophosphate	0.1	1.00	1.00
	1.0	1.06	1.0

The compounds at the indicated final concentrations were added and ciliary activity was measured as described in Fig. 1. The results are averages of the maximal enhancements observed after the addition of the compound. Number of experiments was 20 for ATP and 10–12 for the other compounds. The SE of these averages was up to 20%.

signed to test the effect of ion concentrations were performed only in Ringer solution due to inability to make the necessary changes in the commercial medium. Moreover, in order to prevent any transient effects on the ciliary motility, the tissue was preincubated with the test solution for 30 min before the experiment was started.

Results

Effect of Extracellular ATP and Related Compounds on Ciliary Activity

The effect of external ATP and AMP-PNP on the ciliary frequency is shown in Fig. 1. The two nucleotides enhanced the ciliary frequency two to threefold within 1 sec, depending on their concentration. In some cases (20–30%) the pronounced and rapid effect of ATP resulted in a disturbed, oscillatory ciliary activity for several seconds following the addition of ATP (*data not shown*). The maximum enhancement is achieved at 10 μM. Further increase in ATP concentration lowers the effects. Figure 1 also shows that the ciliary frequency enhancement decreases with time. The rate of the decrease rises with ATP concentration. Neither the ATP-enhanced ciliary frequency nor its decay depended on ATP hydrolysis because both effects are also obtained with AMP-PNP. The decrease of the enhanced ciliary frequency in the presence of relatively high concentrations of ATP or AMP-PNP may suggest the involvement of two (or more) ATP binding sites which may differ in their affinity, rate of binding and effects on ciliary frequency. The possibility that the decay is due to binding of Ca^{2+} by ATP or AMP-PNP, thereby decreasing its free concentration, is ruled out because the decay was not prevented by the addition of Ca^{2+} (*data not shown*). Moreover, this decrease at the higher concentration, is common to all of the added compounds (Table 1).

An insight into the nature of the nucleotides binding site(s) is obtained by comparing the effect of different added nucleotides, nucleosides, and phosphate compounds on the ciliary frequency and the wave velocity in the direction of the effective stroke (Table 1). The triphosphate nucleotides (ATP, AMP-PNP and CTP) are more effective than the diphosphate nucleotides (Ovadyahu et al., 1988), monophosphate nucleotide (AMP), and adenosine.

This suggests that the triphosphate moiety is important for nucleotide binding. This suggestion is supported by the fact that tripolyphosphate, but not pyrophosphate, is also effective, although less than ATP. Recently it was found that the addition of 0.1 to 1 mM adenosine to cultured rabbit tracheal epithelium depressed the ciliary frequency by 20–25% (Tamaoki, Kondo & Takizawa, 1989). The authors, however, were not sure if this effect is directly connected to adenosine-ciliated cell interaction or brought about through intermediate cells. Our result indicates that in tissue cultures limited only to ciliary- and mucus-producing cells (Nevo, Weisman & Sadé, 1975), adenosine enhances ciliary activity. It thus appears that both the base and the phosphate residues are involved in ATP binding.

AMP was found to be less effective than adenosine (Table 1). This can possibly imply an existence of a specific adenosine receptor (Burnstock, 1976) in addition to a receptor for ATP. Adenosine receptors appear in smooth muscle of trachea (Christie & Satchell, 1980). It might be that in ciliary cells of frog esophagus there are two types of receptors, adenosine receptors and ATP receptors. Two types of receptors might appear on the same membrane as was found in the case of smooth muscles of the digestive system (Brown & Burnstock, 1981).

The wave velocity of the metachronal wave is determined by the product of frequency and wavelength. It is directly proportional to the rate of transport by the ciliary systems (Priel, 1987) which is an important physiological feature of water- and mucus-transporting cilia. The nucleotides and other compounds which influence ciliary frequency enhance the wave velocity in the direction of the effective stroke even more, because both frequency and wavelength are enhanced by these compounds. The optimal concentration of the enhancers is 0.1 mM while at higher or lower concentrations the wave velocity decreases (Table 1).

Involvement of Extracellular Ca^{2+} in Ciliary Stimulation by ATP

In the present work, measurement of ciliary stimulation by extracellular ATP was examined over a wide range of free Ca^{2+} concentrations. As can be seen (Fig. 2*A*), ciliary stimulation by ATP depends on free extracellular calcium concentration. In the 10^{-7} to 5×10^{-4} M range of free Ca^{2+} concentration the stimulatory effect of ATP is not observed. A narrow transition zone exists, followed by a plateau which represents the maximum enhancement of ciliary frequency by extracellular ATP. It is worth noticing that even at a quite high extracellular free calcium

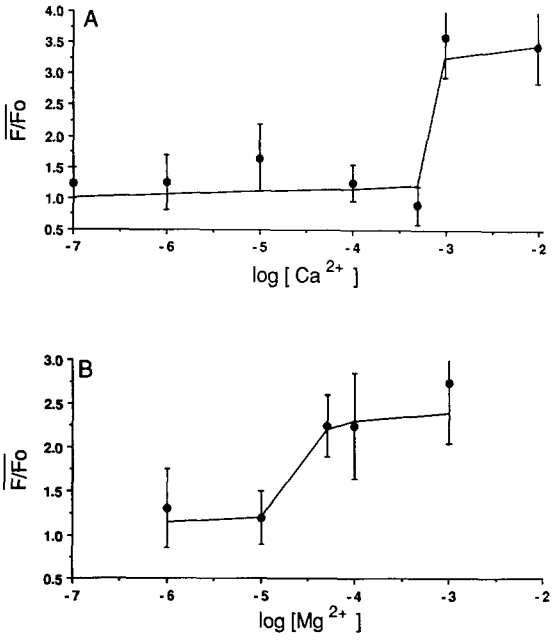

Fig. 2. The enhancement factor (F/F_0) after addition of 10 μM of extracellular ATP as a function of: (*A*) extracellular Ca^{2+} concentration [Mg^{2+}] = 0 and (*B*) extracellular Mg^{2+} concentration, [Ca^{2+}] = 10^{-6} M. The ATP was added after a preincubation in a given solution for 30 min. The enhancement factor given is an average over 5–10 cells from at least two different animals at each ion concentration; bars represent the SE.

concentration (10^{-4} M), three orders of magnitude higher than the intracellular calcium concentration, there is no stimulation by extracellular ATP. In order to check the specificity of the ATP effect to calcium, the exogenous calcium was replaced by magnesium. The free Ca^{2+} concentration in this experiment was quite low (10^{-6} M). It was found that, in principle, magnesium can replace calcium and produce an effect similar to that of Ca^{2+} (Fig. 2*B*). However, the transition zone occurs at a concentration of free Mg^{2+} lower by one order of magnitude than that of free Ca^{2+}. Furthermore, it seems that the enhancement by extracellular ATP of ciliary frequency in the plateau region is a bit lower in the presence of Mg^{2+} as compared to plateau values in the presence of Ca^{2+} (*compare* Fig. 2*A* and *B*).

The possible involvement of Ca^{2+} channels in the enhancement of ciliary frequency by ATP was studied by testing the effects of several specific inhibitors on these channels. Verapamil and nitrendipine at a concentration range of 10^{-6} to 10^{-5} M are known as specific blockers of voltage-gated Ca^{2+} channels (Hille, 1984; Ruff, 1986; Hosey & Lazdunski, 1988). The effect of these blockers on ciliary enhancement by extracellular ATP was tested over a relatively wide range of blocker concentrations,

Table 2. Effect of voltage-dependent Ca^{2+} channel blockers on the enhancement of ciliary frequency by 10 μM extracellular ATP

Blocker	Concentration (μM)	F_{max}/F_0
Ni^{2+}	0.5	2.9
	5	3.7
	50	2.6
	500	2.3
	5000	2.9
La^{3+}	600	2.7
Verapamil	10	2.2
	50	1.5
	100	1.17
Nitrendipine	0.1	2.2
	1	3.0
	10	3.1
	100	3.3

The tissues were preincubated with the blocker for 30 min prior to the addition of ATP. The given factor of enhancement (F_{max}/F_0) was an average over 5–10 cells, with a SE up to 20%.

while the concentration of extracellular ATP was 10 μM (at which a maximum enhancement is achieved; *see* Fig. 1). Table 2 shows that verapamil but not nitredipine may be an inhibitor of the ATP effect. The specificity of verapamil as a potent blocker of voltage-gated Ca^{2+} channel was proven to be at the micromolar concentration range. As can be seen (Table 2) verapamil does not have an inhibitory effect in this range of concentrations. Moreover, recently there is a growing awareness that at a higher concentration verapamil may interact with ion pumps and inhibit different channels, as for example Na^+ (Bondy, 1978; Esau, 1988). Therefore, inhibition of the extracellular ATP effect by 0.1 mM verapamil cannot be used as a proof of the involvement of voltage-gated Ca^{2+} channels. In order to clarify whether Ca^{2+} channels are involved in our system, experiments were conducted with inorganic inhibitors of voltage-gated Ca^{2+} channels (Ruff, 1986). It was found that Ni^{2+} over quite a wide range of concentrations and La^{3+} at a relatively high concentration do not inhibit the effect of extracellular ATP (Table 2). Recently, it was reported (Villalon et al., 1989) that La^{3+} (0.6 mM) inhibits the effect of extracellular ATP on mammalian ciliated cells. The discrepancy between the results may arise because of the differences between the two preparations or because of the solutions in which the experiments were conducted.

Involvement of K^+ Channels in Exogenous ATP Stimulation

The possible involvement of K^+ channels in the enhancement of ciliary frequency by ATP was studied

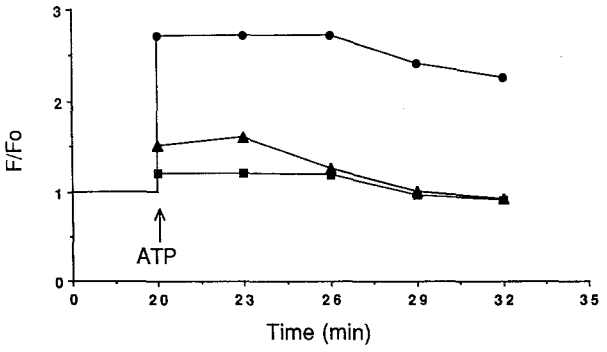

Fig. 3. Typical time courses of the effect of quinidine on the ATP-stimulated ciliary frequency. Solution (100 μl) containing quinidine and ATP was added to the final concentrations of: 1 mM quinidine with 10 μM of ATP (■), 50 μM quinidine and 10 μM ATP (▲), and ATP 10 μM (●). Quinidine was dissolved in ethanol. The final concentration of ethanol in the assay medium was not more than 0.5%.

Table 3. The inhibitory effect of quinidine in the presence of various concentrations of ATP and AMP-PNP

Nucleotide	% of inhibition	
	Quinidine (50 μM)	Quinidine (1 mM)
ATP, 10 μM	65	88
ATP, 0.1 mM	34	57
ATP, 1 mM	24	49
AMP-PNP, 0.1 mM	—	50

The experiments were performed as those described in Fig. 1 except that the indicated nucleotide was added in the absence or the presence of quinidine. Percent of inhibition was calculated from the ratio $(F - F_0)$ ATP + quinidine/$(F - F_0)$ ATP.

by testing the effects of two specific inhibitors of these channels. Quinidine, in the millimolar concentration range, is a K^+ channel blocker for K^+ channels activated by Ca^{2+} (Hille, 1984; Ruff, 1986). In smooth muscle, quinidine is a powerful inhibitor of extracellular ATP effect (Burnstock, 1972, 1976). The effect of two concentrations of quinidine on ciliary frequency in the presence of extracellular ATP is shown in Fig. 3. As can be seen, 1 mM of quinidine inhibited almost completely the frequency enhancement while 50 μM partially inhibited this enhancement. The same inhibitory effect was achieved by either simultaneous addition of ATP and quinidine or by adding the blocker after the ATP (*data not shown*).

Table 3 shows that the inhibitory effect of quinidine is dependent on ATP concentration and decreases with increasing ATP concentration. As ex-

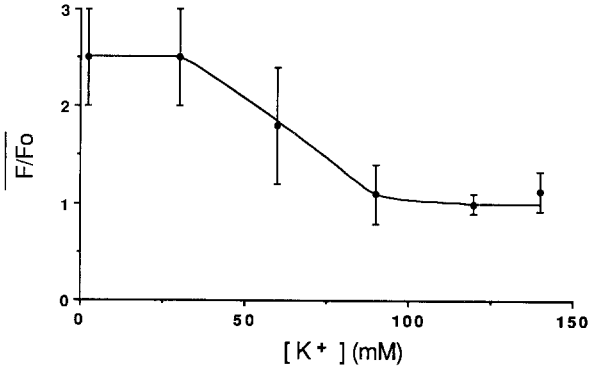

Fig. 4. The enhancement factor (F/F_0) after addition of 10 μM of extracellular ATP, as a function of varying concentrations of extracellular K^+ ions. The osmolarity was kept constant by replacing Na^+ with K^+. ATP was added after preincubation of the tissue with a given K^+ concentration for 30 min. Averages are of 5–10 cells at each concentration; bars represent the SE.

pected, quinidine also inhibited the stimulatory effect of AMP-PNP. Quinidine has no effect on the ciliary frequency obtained in the absence of ATP. Tetraethylammonium (TEA), another blocker of K^+ channels (Hille, 1984; Ruff, 1986) in the commonly used range of 1–20 mM concentrations, has no inhibitory effect on the ciliary activity enhancement produced by ATP (*data not shown*).

In order to investigate the influence of K^+ flux through the cell membrane on the stimulatory effect of ATP, the extracellular concentration of K^+ was gradually raised from 2.5 to 140 mM. To keep the osmolarity constant, the Na^+ ions in the Ringer solution were replaced by K^+ ions accordingly. Figure 4 shows that raising the external K^+ concentration lowers the enhancement of ciliary motility by extracellular ATP in a concentration-dependent manner. Already at a concentration of 90 mM of K^+ the extracellular ATP effect is completely blocked (Fig. 4). It should be mentioned that immediately after replacing the medium or Ringer by a solution with higher K^+ concentration, transient effects on ciliary motility occur. However, within 30 min these transient effects vanish and normal ciliary activity is restored for a long time at all K^+ concentrations. Therefore, all the experiments with K^+ ions (Fig. 4), as with most of the other blockers, included preincubation with the testing solution for at least 30 min.

Discussion

The effect of low concentrations of extracellular ATP on the ciliary transport rate and frequency is a well-documented observation (Varhaus & Deyrup, 1953; Usuki, 1959; Murakami et al., 1974; Nelson & Wright, 1974; Ovadyahu et al., 1988; Villalon et al., 1989). However, very little information is available on ATP-interacting site(s) and the mechanism by which ATP produces its effect on ciliary motility. Our basic assumption is that extracellular ATP interacts with the ciliary cell membrane. Although protein kinases utilizing extracellular ATP have been found in several cell types (Krebs, 1986) the fact that AMP-PNP is also effective suggests that the effect of ATP is not mediated through protein phosphorylation or any other reaction involving its hydrolysis. The triphosphate and the base moieties are important for bringing about the ciliary response, (Table 1).

Extracellular ATP acts as a neurotransmitter in a variety of biological systems (Burnstock, 1972, 1976). Since ciliary cells belong to a category of excitable cells, their interaction with exogenous ATP may be described by the following sequence of events which commonly occur in cases of neurotransmitter signalling: There is binding of the neurotransmitter followed by a change in action potential which causes a depolarization of the membrane. Voltage-sensitive calcium channels are then opened by membrane depolarization, admitting calcium into the cell. Rise of intracellular Ca^{2+} concentration (within certain limits) causes an enhancement of the ciliary beating, as was shown in ciliated epithelia by ionophoric injection of Ca^{2+} (Verdugo, Rumery & Lee, 1977; Girard & Kennedy, 1986) and in demembranated protozoa (Andrivon, 1988; Machemer, 1990). However, this appealing model cannot explain our findings. According to this model the potent inhibitors of voltage-sensitive calcium channels should block the extracellular ATP effect, but they do not (Table 2). Moreover, the dependence of the ATP effect on extracellular free Ca^{2+} concentration (Fig. 2) indicates that there is no flux of Ca^{2+} ions from the outside into the cell. Even at 10^{-4} M extracellular free Ca^{2+}, which is about three orders of magnitude higher than the Ca^{2+} concentration inside the cell, there is no effect. In addition, ATP stimulates the ciliary beat frequency when extracellular Ca^{2+} is replaced by Mg^{2+} (Fig. 2*B*). This supports the argument that Ca^{2+} is involved in the ATP effect but most probably not by entering the cell through voltage-gated Ca^{2+} channels. On the other hand, there is a variety of calcium-binding proteins in cell membranes or in cytosol which mediate the Ca^{2+} second-messenger signalling or regulate the intracellular level of free Ca^{2+}. Some of them require Ca^{2+} as a cofactor for their activity (Salomon, 1990). Magnesium ions may compete or replace calcium ions as cofactors of these proteins and produce a similar effect. A critical concentration of the divalent ion

(calcium or magnesium) is usually necessary, similar to the dependence found by us (Fig. 2). Another possibility is that exogenous ATP is effective in a form of complex with a divalent cation. The fact that extracellular Ca^{2+} can be replaced by Mg^{2+} rules out the possibility that extracellular ATP opens a nonspecific, low conducting cation channel through which Ca^{2+} as well as Na^+ and Mg^{2+} can pass (Benham & Tsien, 1987; Krishtal et al., 1988). We therefore conclude that exogenous calcium at a relatively high concentration is needed as a cofactor to the ATP receptor or as a ligand for the ATP ion.

It was shown that extracellular ATP activated receptor-mediated calcium mobilization in several systems (Kung & Saimi, 1982; Dubyak, 1986; Horstman et al., 1986; Fine, Cole & Davidson, 1989; Gonzalez et al., 1989). A rise in free cytosolic calcium in some cases is known to increase the frequency of ciliary beating (Andrivon, 1988; Machemer, 1990). Therefore, if intracellular calcium concentration is elevated because of ATP stimulation, the first consequence might be a rapid increase of the frequency. In addition, if Ca^{2+}-activated K^+ channels are found in the cellular membrane, they will probably be activated to the open state because of the rise in the concentration of free cytosolic calcium.

We have found in the present work that quinidine, which in millimolar concentration is a specific inhibitor of the Ca^{2+}-activated K^+ channels (Hille, 1984; Petersen & Maruyama, 1984; Ruff, 1986), caused an inhibition of ATP enhancement of the ciliary activity (Fig. 3). This suggests that these channels are open in the presence of ATP. The same results were also found in smooth muscle (Burnstock, 1976) and in parotid acinar cells (Gallacher, 1982). Also quinin, the stereoisomer of quinidine, blocked the K^+ permeability in isolated hepatocytes in the presence of ATP (Burgess et al., 1979). There appear to be three distinct types of K^+ channels activated by Ca^{2+} which differ from each other in K^+ conductance, in membrane voltage sensitivity and in their susceptibility to specific blockers (Petersen & Maruyama, 1984; Ruff, 1986). Only one of them is blocked by TEA (Ruff, 1986). Our result that TEA has no influence on the ATP effect and that quinidine is a potent inhibitor of the ATP effect supports this subclassification.

Ca^{2+}-activated K^+ channels play a major role in regulating changes in membrane potential (Petersen & Maruyama, 1984; Ruff, 1986). Our results show (Fig. 4) that with increasing external concentration of K^+ (keeping the osmolarity constant) the stimulatory effect of ATP on ciliary motility is decreased in a dose-dependent manner and is completely prevented when extracellular K^+ concentration is above 90 mM. This indicates that in the presence of extracellular ATP these channels, in their open conformation, allow K^+ to leak out of the cells. It is reasonable to assume that this K^+ efflux causes hyperpolarization of the membrane and that the ATP enhancement of the ciliary activity is a result of this change of the membrane potential. When the concentration gradient of K^+ is lowered by external addition of K^+, the change of membrane potential due to opening of K^+ channels decreases accordingly, thereby lowering the stimulating effect of extracellular ATP. In other cell types, it was shown that external addition of ATP caused hyperpolarization (Gallacher, 1982; Okada et al., 1984). In ciliated cells it was found that changes (rise and fall) of the membrane potential raise the ciliary frequency (Eckert, 1972; De Peyer & Machemer, 1982; Den Hertog, Nelemans & Van den Akker, 1989; Molleman, Nelemans & Den Hertog, 1989).

The necessity of involvement of hyperpolarization of the membrane to achieve and maintain the ciliary enhancement by extracellular ATP for a relatively long time was demonstrated by replacing exogenous Na^+ by K^+ (Fig. 4). Moreover, it was shown, that this hyperpolarization was achieved via Ca^{2+}-activated K^+ channels (Fig. 3 and Table 3). The existence of Ca^{2+}-activated K^+ channels in ciliary systems was shown previously (Brehm, Dunlop & Eckert, 1978; Satow & Kung, 1980; Adoutte et al., 1981; Kung & Saimi, 1982; Saimi et al., 1983). However, their main physiological role in these systems was believed to be the return of the membrane potential to its resting state after depolarization by Ca^{2+} response, while in our case these channels are used for hyperpolarization of the membrane from the resting potential as a part of the ciliary stimulation. The mechanism by which the hyperpolarization of the membrane causes the elevation of the frequency during the stimulation by extracellular ATP is still unclear.

Based on our findings, the following mechanism may be suggested (Fig. 5): extracellular ATP interacts with a receptor that resides in the plasma membrane of the cell. This interaction involves Ca^{2+} ions. This interaction starts a cascade of events, ending in increased intracellular free Ca^{2+} concentration released from internal stores. When the calcium concentration within the cell increases, two events occur: (i) The Ca^{2+}-activated K^+ channels open, allowing K^+efflux out of the cell according to the concentration gradient. This would lead to the change of the cell membrane potential to a more negative value. (ii) The ciliary beat frequency increases.

This model predicts that applying extracellular ATP to a ciliated cell will cause: (i) a rise of intracellular Ca^{2+} and (ii) hyperpolarization of the ciliary

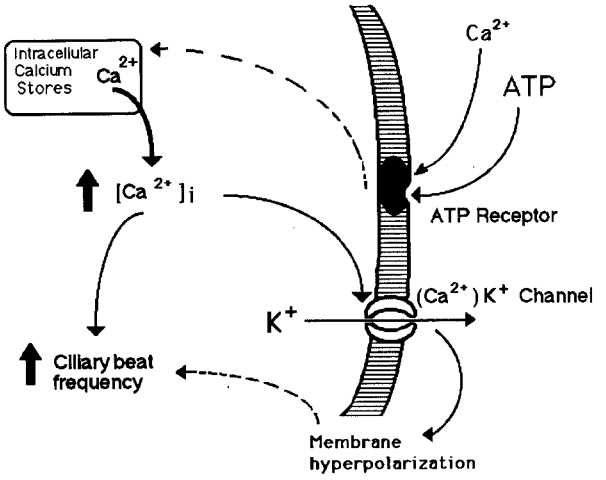

Fig. 5. A schematic diagram of the suggested model: extracellular ATP and a Ca^{2+}-activated membrane receptor initiate a series of events leading to mobilization of intracellular Ca^{2+}. Increase of free Ca^{2+} concentration in the cell causes an increase in ciliary beat frequency and opens calcium-activated K^+ channels. Opening of K^+ (Ca^{2+}) channels allows K^+ efflux according to its concentration gradient, thereby, decreasing the membrane potential which further increases ciliary frequency.

membrane potential. Indeed, it was found that after applying extracellular ATP, the intracellular Ca^{2+} concentration increases by almost a factor of two (A. Korngreen & Z. Priel, *unpublished results*). Similar results were recently published (Villalon et al., 1989). Furthermore, measurements of resting membrane potential by microelectrodes revealed that extracellular ATP introduces hyperpolarization in a dose-response manner, reaching a maximum at 10^{-5} M ATP (A. Tarsiuk, M. Bar-Shimon, R. Grossman & Z. Priel, *unpublished results*).

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