Extracellular ATP Induces Hyperpolarization and Motility Stimulation of Ciliary Cells

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ABSTRACT Cellular membrane potential and ciliary motility were examined in tissue cultures prepared from frog palate and esophagus epithelia. Addition of micromolar concentrations of extracellular ATP caused membrane hyperpolarization and enhanced the beat frequency. These two effects of ATP were 1) dose dependent, reaching a maximum at 10 μM ATP; 2) dependent on the presence of extracellular Ca^{2+} or Mg^{2+}; 3) insensitive to inhibitors of voltage-gated calcium channels; 4) abolished after depleting the intracellular Ca^{2+} stores with thapsigargin; 5) attenuated by quinidine (1 mM), Cs^{+} (5–20 mM), and replacement of extracellular Na^{+} by K^{+}; 6) insensitive to charybdotoxin (5–20 nM), TEA (1–20 μM), and apamin (0.1–1 μM); 7) independent of initial membrane potential; and 8) unaffected by amiloride. In addition, extracellular ATP induced an appreciable rise in intracellular Ca^{2+}. Addition of thapsigargin caused an initial enhancement of the ciliary beat frequency and membrane hyperpolarization. These results strongly suggest the involvement of calcium-dependent potassium channels in the response to ATP. The results show that moderate hyperpolarization is closely associated with a sustained enhancement of ciliary beating by extracellular ATP.

INTRODUCTION

Ciliary cells can respond to a variety of mechanical, electrical, chemical, and hormonal stimuli by altering their pattern of beating. The pronounced effect of extracellular ATP on ciliary transport rate and beat frequency is well documented (Varhaus and Deyrup, 1953; Usuki, 1959; Murakami et al., 1974; Nelson and Wright, 1974). However, the physiological importance of extracellular ATP is not well understood. A detailed examination of most of the metachronal wave parameters as a function of extracellular ATP was performed in our laboratory (Ovadyahu et al., 1988; Gheber and Priel, 1994). These studies revealed that the ciliary beating frequency, amplitude, metachronal wavelength, and velocity in the direction of the effective stroke are influenced considerably by application of 1–10 μM extracellular ATP.

Recently, we investigated the cellular events underlying the stimulation of the ciliary beat frequency by extracellular ATP. Based on the findings, we proposed that extracellular ATP interacts with a membrane receptor and induces intracellular calcium mobilization from intracellular stores (Weiss et al., 1992). The increase in cytosolic free Ca^{2+} concentration induces the opening of calcium-activated K^{+} channels, leading to membrane hyperpolarization. In this model both Ca^{2+} increase and the membrane hyperpolarization contribute to the strong and prolonged enhancement of the ciliary beat frequency induced by ATP. This model predicts that the addition of extracellular ATP to ciliated cells will cause a rise in cytoplasmic Ca^{2+} released from internal stores and a change in membrane potential toward more negative values. A rise in intracellular calcium concentration was observed in several cultured mucociliary epithelia (Villalon et al., 1989; Hansen et al., 1993; Korngreen and Priel, 1993, 1994) including the present work; however, the change in membrane potential is shown for the first time in this study.

MATERIALS AND METHODS

Preparation and solutions

Experiments were carried out on monolayer tissue cultures grown from excised frog (*Rana ridibunda*) palate and esophagus using the procedure described previously (Eshel et al., 1985). The Ringer's 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, at pH 7.2. Charybdotoxin was purchased from Alomone Labs (Jerusalem, Israel). Fura-2/AM was purchased from Molecular Probes (Eugene, OR). All other drugs and reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Solutions with a low concentration of Ca^{2+} and Mg^{2+} were prepared by adding known amounts of Ca^{2+} and Mg^{2+} and EGTA to Ca^{2+}-free Ringer's solution. The total concentration of EGTA did not exceed 0.5 mM. The free ion concentration was calculated using a computer program previously described (Jean and Klee, 1986).

Experiments were carried out on 4–16-day cultures. Before the measurements, the bathing medium was washed several times with Ringer's solution to deplete the amount of mucus as much as possible. To avoid transient effects, the tissue cultures were allowed to equilibrate with the solutions for at least 20 min before starting measurements.

Electrical measurements

Electrical measurements were performed only on cells in dense ciliary regions such as were usually observed 100–500 μm away from the original explant. Intracellular membrane potential (V) was measured by means of a 25–50 MΩ microelectrode, filled with 4 M K-acetate, connected to a high impedance electrometer (Model 707, WP Instruments, New Haven, CT) via an Ag-AgCl wire. The voltage was constantly monitored using an oscilloscope. It was extremely difficult to record stable intracellular recordings for more than a few minutes because of the small size of the ciliary epithelial

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cells (10–20 × 9–12 × 3–5 μm). Therefore, the criterion for steady electrical measurements was a stable voltage between 20 and 40 mV (within ±2 mV) lasting for ~30–60 s. Membrane potentials were referenced to the potential recorded after withdrawal of the microelectrode from the cell. Generally, this potential was similar to the ground potential recorded before impaling the cell. Measurements from ~15–20 cells were averaged under controlled conditions. The culture was then treated with the test solution, and the measurements were repeated. Finally, the culture was washed again with Ringer's solution and the resting membrane potential was measured. The most important criterion for accepting the results was the reversibility of the voltage after replacement of the test solution by the Ringer's solution. The same experiment was repeated on two to four different cultures, and the results were averaged.

Measurements of ciliary beating

Simultaneous measurements of scattered light from two points on the monolayer of ciliary epithelium was performed as previously described (Eshel and Priel, 1987). This method enables the measurement of ciliary beat frequency, and other important parameters of ciliary activity such as metachronal wavelength and velocity (Gheber and Priel, 1989, 1990, 1994; Priel, 1987).

To measure the effect of a given treatment on ciliary activity the following procedure was adopted. 1) After a preincubation period of 20–30 min in Ringer's solution, several measurements of the ciliary beat frequency, 5–40 s each, were recorded from the ciliary area. The average of these measurements served as the control frequency for a given experiment (F_o). The last control measurement was performed just before the addition of the stimulant. 2) A small volume of solution of the compound to be tested was added to a constant volume of Ringer's solution, and its effect on the ciliary beat frequency was measured over time. 3) The values of the average ciliary beat frequency over 10–30 s (F), obtained under the influence of a given substance at each given time, were normalized to the control frequency (F/F_o). The normalized maximal frequency (F_m/F_o) represents the degree of enhancement of the ciliary beat frequency under a given condition.

Each experiment was carried out on five to twenty tissue cultures from two to three animals. Performing the entire experiment on the same ciliary cell greatly enhanced the accuracy of the results. To rule out the possibility that the addition of solution mechanically stimulated the cilia, control experiments were performed in which an equivalent amount of Ringer's solution was added. No changes in ciliary activity were detected in these control experiments.

Measurements of $[Ca^{2+}]_i$

The intracellular free calcium $[Ca^{2+}]_i$ was measured, from single ciliary cells, using the fluorescence indicator fura-2, as previously described (Korngreen and Priel, 1993, 1994). Briefly, the cells were alternatively epiilluminated with light at wavelengths of 340, 360, and 380 nm. The emission fluorescence at 510 nm was collected by a photomultiplier positioned on the photo-eyepiece of the microscope (Axioskope, Carl Zeiss, Germany). The values of the 340/380 fluorescence ratio and the 360 intensity were stored on computer at 2-s intervals. This allowed both the intracellular calcium concentration and the total concentration of fura-2 in the cell to be monitored.

Cells were loaded with 5 μM fura-2/AM in 1 ml growth medium for 30 min at room temperature and washed three times with Ringer's solution. Because of the very low activity of the esterase responsible for the cleavage of the acetoxymethyl protecting groups of the fura-2, we encountered a high rate of failure in loading the fura-2 into the cells. Therefore, in all the experiments, the fluorescence of the dye with a 360-nm excitation wavelength was monitored. Experiments were carried out only on cells that exhibited a high and stable fluorescence at this wavelength.

RESULTS

Extracellular ATP hyperpolarized ciliary epithelial cells (Fig. 1 *A*) in a dose-dependent manner. A maximal effect was observed with 10 μM ATP, which hyperpolarized the membrane by a factor of 1.26 ± 0.05 (from −28 to −35 mV). The dependence of the membrane potential and ciliary beat frequency on extracellular ATP concentration is similar (Fig. 1 *B*). Both effects reach a maximum at 10 μM ATP and diminish at higher concentration. Neither the ATP-induced hyperpolarization nor the enhancement of ciliary activity requires ATP hydrolysis since similar effects were observed with AMP-PNP, a nonhydrolyzable analog of ATP (not shown).

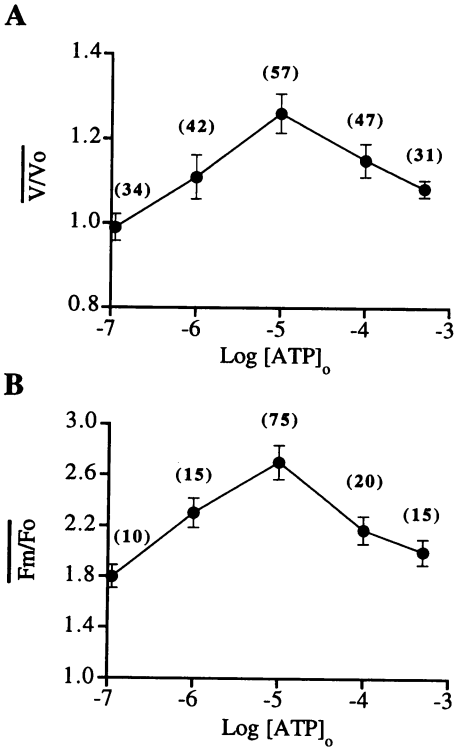

FIGURE 1 The effect of ATP on membrane potential and on ciliary beat frequency. (*A*) Normalized membrane potential (*V/Vo*) as a function of extracellular ATP concentration. Hyperpolarization is defined as an increase in the membrane potential. (*B*) Normalized maximal frequency (*Fm/Fo*) as a function of ATP concentration. Bars represent the SE values, the number of experiments at each concentration indicated in parentheses.

To investigate the events initiated by the interaction of ATP with the membrane receptor, we measured the intracellular calcium concentration. Addition of extracellular ATP (10 μM) to the cells (Fig. 2 *A*) caused a rapid rise in $[Ca^{2+}]_i$, which decayed, in ~4 min, to a new sustained level that was higher than basal (n = 5). Fig. 2 *B* shows that the relative intensity of fura-2, displayed as percentage over the autofluorescence, decayed only by 5% during the whole experiment. This indicates that the fura-2 did not leak out of the cell and was not photobleached.

To determine the source of the rise in $[Ca^{2+}]_i$ we used the sarcoplasmic Ca^{2+}-ATPase inhibitor thapsigargin to deplete the intracellular calcium stores. Fig. 3 displays the effect of 1 μM thapsigargin on membrane potential and on ciliary beat frequency. Thapsigargin induced a transient membrane hyperpolarization (Fig. 3 *A*) and a transient rise in ciliary beat frequency (Fig. 3 *B*). Both effects probably result from

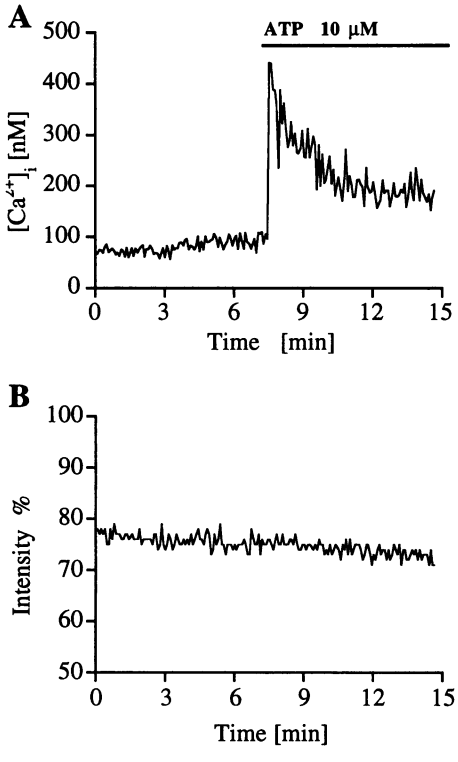

FIGURE 2 A typical effect of extracellular ATP on intracellular calcium concentration $[Ca^{2+}]_i$. (*A*) Change in $[Ca^{2+}]_i$ in response to 10 μM of ATP. The horizontal bar indicates the presence of ATP. (*B*) The total fluorescence of the dye fura-2, measured with an excitation wavelength of 360 nm given as percentage over the background fluorescence.

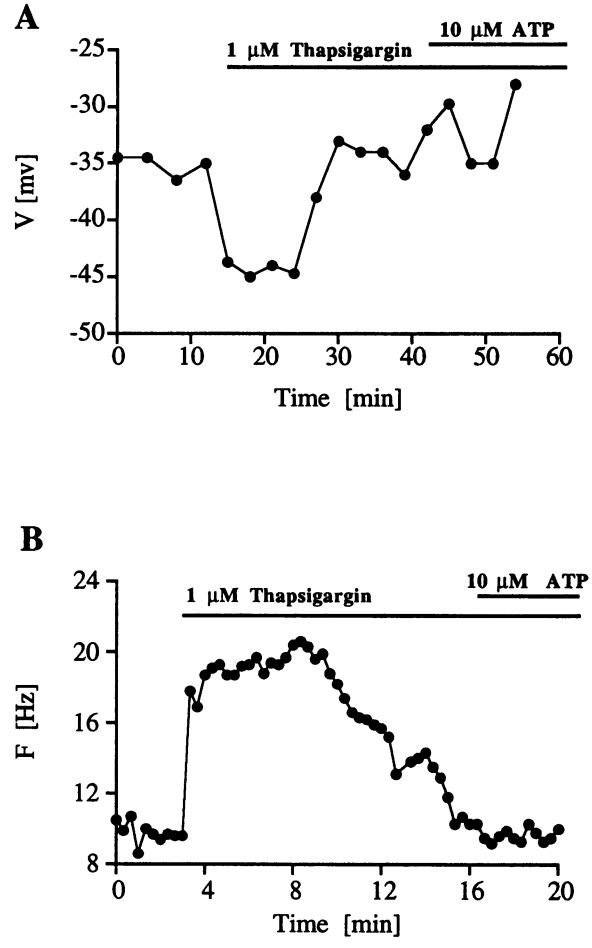

FIGURE 3 The effect of 1 μM thapsigargin on membrane potential (*A*) and ciliary beat frequency (*B*). Depletion of the calcium stores inhibits both the ATP-induced rise in ciliary beat frequency and the ATP-induced membrane hyperpolarization. The final DMSO concentration in the test solution did not exceed 0.2% (v/v). (*A*) Time course of the effect of thapsigargin on membrane potentials (*V*) measured on the same tissue culture. Each point represents an average over 30–60 s of potential recording from three to four cultured ciliary cells; SE of all averaged seconds was up to 2%. (*B*) Time course of the effect of thapsigargin on ciliary beat frequency (*F*) measured on the same ciliary cell. Each point represents average frequency over 10 subsequently measured seconds; SE = ≤ 1%.

thapsigargin-induced calcium release from internal stores. After these effects decayed, 10 μM of extracellular ATP failed to enhance ciliary beat frequency (n = 20) nor to induce membrane hyperpolarization (n = 56) (Fig. 3). These effects of thapsigargin do not result from the solvent DMSO since the final concentration of DMSO in these experiments was 0.2%. At such concentration DMSO has no effect on the ciliary beat frequency nor on the membrane potential (data not shown). The similarity of the effects of ATP and thapsigargin and the inefficiency of ATP after depleting the Ca^{2+} stores strongly suggest that extracellular ATP causes a rapid release of calcium from internal stores in these cells and that the rise in intracellular calcium is directly related to enhanced ciliary beat frequency and membrane hyperpolarization.

It was previously shown (Weiss et al., 1992) that the enhancement of ciliary frequency by extracellular ATP depends in a similar manner on free exogenous Ca^{2+} or Mg^{2+} concentrations. Fig. 4 *A* shows that membrane hyperpolarization depends on the extracellular free calcium concentration. At extracellular Ca^{2+} concentrations lower than $5 \cdot 10^{-4}$ M ATP did not induce membrane hyperpolarization. A narrow transition zone of extracellular Ca^{2+} concentration exists ($5 \cdot 10^{-4}$–$2 \cdot 10^{-3}$ M) at which the effect of ATP gradually increased reaching an apparent maximum at ~$2 \cdot 10^{-3}$ M. To check whether the effect of ATP depends exclusively on extracellular Ca^{2+}, calcium was replaced by magnesium. The free Ca^{2+} concentration in these experiments was on the order of 10^{-6} M. It was found that, in principle, magnesium can substitute for calcium, producing an effect similar to that of Ca^{2+} (Fig. 4 *B*). These data show that exogenous calcium at a relatively high concentration is required (probably as a cofactor) for the ATP effects on both epithelial membrane potential and ciliary motility.

The effect of ATP was also examined in the presence of potent Ca^{2+} channel blockers. Addition of verapamil, a blocker of voltage-gated Ca^{2+} channels, at a concentration indicating the upper limit of its specificity (10 μM), influenced neither the ATP-induced membrane hyperpolarization nor the resting membrane potential (not shown). Increasing the verapamil concentration to 50 μM (which is considerably above the range of its specific effect as a blocker of voltage-gated Ca^{2+} channels) caused a small but significant membrane depolarization, but did not interfere with the

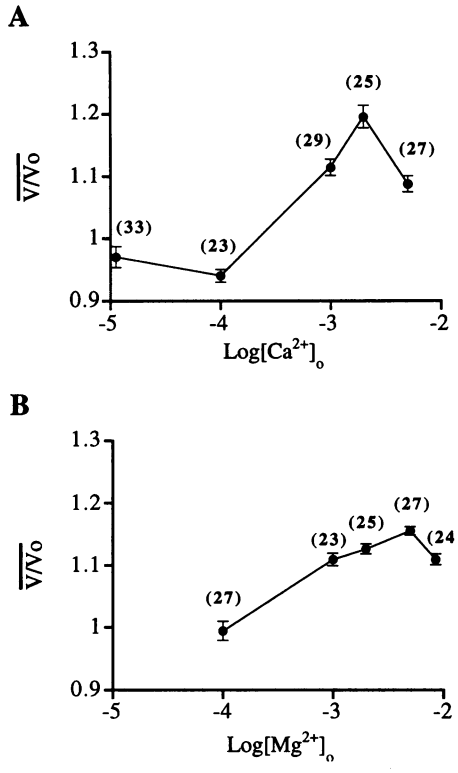

FIGURE 4 Effect of extracellular calcium (*A*) or magnesium (*B*) on normalized membrane potential response (*V/Vo*) to exogenous ATP (10 μM). The concentration of Ca^{2+} or Mg^{2+} is given in units of mol/l. Normalization of membrane potential is as in Fig. 3. Bars represent SE; the number of experiments is indicated in parentheses.

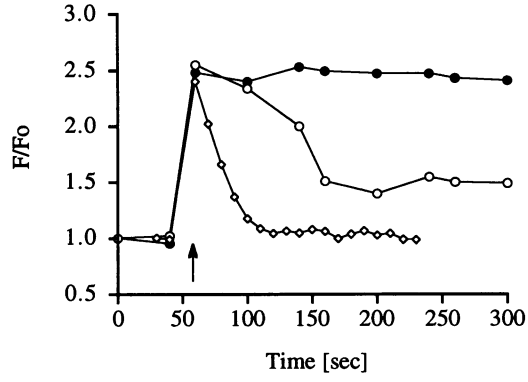

FIGURE 5 Time course of ATP-induced ciliary beat frequency enhancement in the presence of K^+-channels inhibitors. 100 μl of ATP solution was added (indicated by an arrow) to 900 μl of Ringer's solution to reach a final concentration of 10 μM (●), Ringer's solution containing 20 mM Cs^+ (○), and 1 mM quinidine (◇). Each point represents an average of 10 s, subsequently measured. SE ≤ 2%.

extracellular ATP effect (not shown). Similarly Cd^{2+} (0.1 mM) and Ni^{2+}, (up to 5 mM), known calcium channel blockers, failed to produce any change in the ATP response (not shown). It seems, therefore, that Ca^{2+} influx through voltage-gated Ca^{2+} channels is not responsible for the ATP-induced hyperpolarization.

The effects of extracellular ATP on both membrane potential and ciliary beat frequency were inhibited by blocking potassium channels. The inhibition of ciliary beat frequency was time dependent. The inhibitors hardly affected the initial enhancement of the ciliary beat frequency, but greatly attenuated the sustained level of activity (Fig. 5). Extracellular Cs^+ at relatively high concentrations blocks most known potassium channels. Indeed, 20 mM Cs^+ completely abolished the ATP-induced hyperpolarization (Fig. 6 *A*) and considerably reduced the duration of the enhancement of ciliary frequency induced by extracellular ATP (Figs. 5 and 6 *B*).

Quinidine, a potent inhibitor of calcium-dependent potassium channels, abolished membrane hyperpolarization and suppressed the prolonged ciliary enhancement caused by extracellular ATP (Figs. 5 and 6). Quinidine by itself did not influence the resting membrane potential (not shown). The quinidine block could be removed by washing with normal Ringer's solution. Other potent inhibitors of calcium-activated potassium channels such as charybdotoxin (5–20 nM), apamin (0.1–1 μM), and TEA (1–20 μM) (not shown) influenced neither the enhancement of ciliary frequency nor the membrane hyperpolarization induced by extracellular ATP.

Ba^{2+} is a potent inhibitor of K^+ permeability (Latorre and Miller, 1983; Grygorezyk et al., 1989). Ba^{2+} at a concentration of 5 mM depolarized the ciliary cell membrane (Table 1). Yet, this depolarization did not diminish the effect of ATP (Fig. 6 *A*). Addition of 5 mM Ba^{2+} to ciliated epithelium in Ringer's solution enhanced ciliary frequency by a factor of 1.6 (not shown). We have recently shown that both membrane depolarization and hyperpolarization enhanced ciliary frequency in the frog cultured epithelium (Bar-Shimon et al., 1992). Therefore, the increase in ciliary beat frequency induced by Ba^{2+} could be due to membrane depolarization. However, 10 μM exogenous ATP in the presence of Ba^{2+} further enhanced ciliary activity to its maximum values (Fig. 6 *B*). It seems, therefore, that neither ATP-induced hyperpolarization nor ciliary enhancement are mediated via potassium channels that are sensitive to 5 mM extracellular Ba^{2+}.

Decreasing the potassium gradient across the membrane by replacing 100 mM of NaCl in the Ringer's solution by 100 mM KCl thereby abolished the prolonged enhancement of ciliary frequency induced by extracellular ATP (see Weiss et al., 1992, and Fig. 6 *B*). As expected, substituting the NaCl with KCl induced a considerable depolarization of the ciliary membrane and prevented extracellular ATP-induced membrane hyperpolarization (see Table 1 and Fig. 6 *A*).

Both changing the KCl concentration and adding Ba^{2+} causes depolarization. Therefore, the question whether the extracellular ATP effect is dependent on the initial membrane potential was explored further. Amiloride, a potent inhibitor of Na^+ channels at a concentration of 1 μM hyperpolarized the ciliary membrane from −28.0 to −38.0 mV (Table 1). Ciliary frequency was increased by amiloride by a factor of 1.8 (Bar-Shimon et al., 1992). Addition of extracellular ATP at a concentration of 10 μM in the presence of amiloride further increased ciliary frequency to its

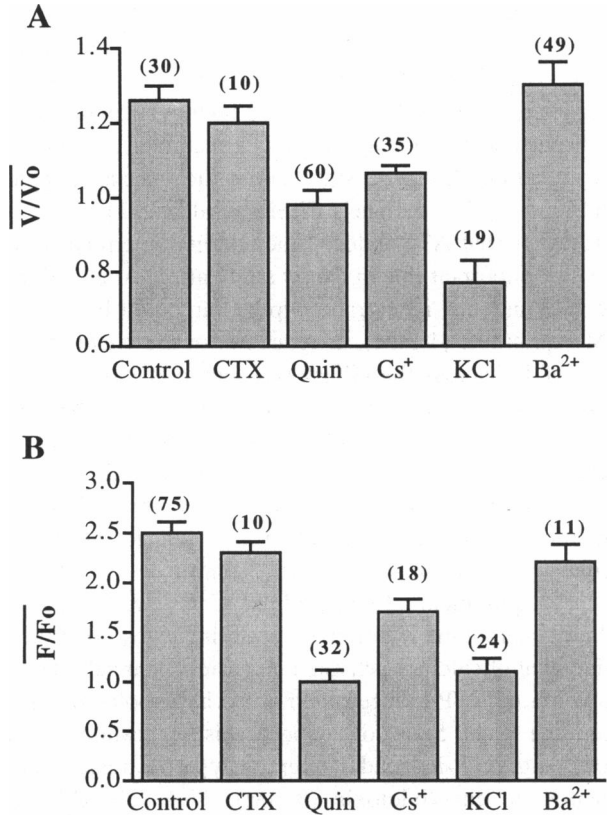

FIGURE 6 Effect of K^+-channel inhibitors on the ATP-induced changes in membrane potential and on ciliary beat frequency. The concentrations of the inhibitors are 5 nM charybdotoxin (CTX); 1 mM quinidine (Quin); 20 mM Cs^+; 100 mM K^+ (100 mM Na^+ removed); 5 mM Ba^{2+}. The extracellular ATP concentration is 10 μM. *Control* represents the addition of ATP to the Ringer's solution. Bars represent the SE values; the number of experiments is indicated in parentheses. (*A*) Average ATP-induced normalized membrane potential (*V/Vo*). The membrane potential obtained after the addition of extracellular ATP (*V*) was normalized to the value of the membrane potential obtained after 20–30 min of preincubation in the indicated conditions (*Vo*). (*B*) Average normalized ciliary beat frequency (*F/Fo*) obtained 3 min after the addition of extracellular ATP to cilia preincubated for 20–30 min in the indicated conditions.

TABLE 1 Membrane potential V as a function of different conditions

Treatment	$V \pm$ SE mV	(n)
Control	-28.0 ± 0.7	(60)
ATP 10 μM	-36.2 ± 0.9	(57)
Amiloride 1 μM	-38.0 ± 2.1	(58)
Cs^+ 20 mM	-25.0 ± 2.1	(27)
Verapamil 10 μM	-27.7 ± 1.1	(33)
CTX 2 nM	-24.9 ± 2.0	(35)
Ba^{2+} 5 mM	-23.8 ± 0.1	(70)
Replacement of 100 mM of Na^+ with K^+	-15.5 ± 2.1	(33)
Replacement of 120 mM Cl^- by SO_4^{2-}	-8.1 ± 2.2	(49)

Experiments at each category were on two to five different cultures.

maximum value (Fig. 7 *A*). As mentioned above, a similar effect was observed when the ciliary membrane was depolarized by Ba^{2+} (Fig. 6). Membrane depolarization was achieved by replacing Cl^- ions in the Ringer's solution by SO_4^{2-} or glucoronate. The membrane potential was depolarized by these two

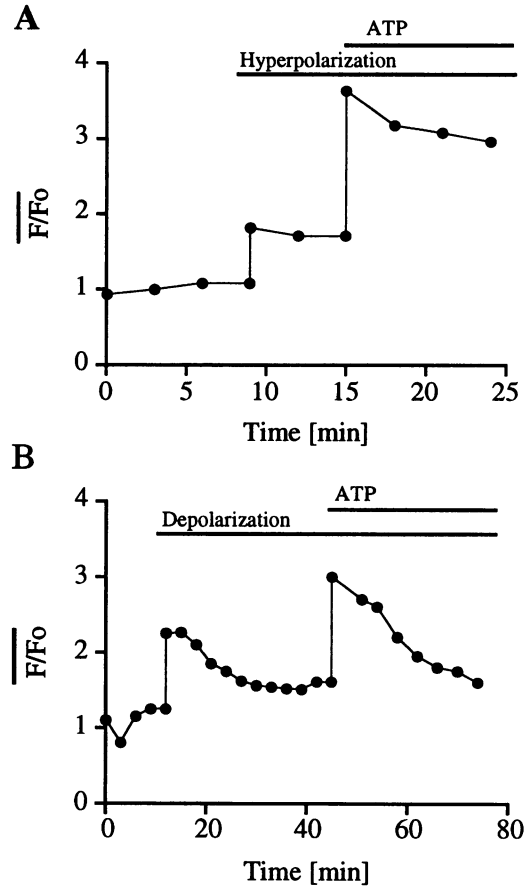

FIGURE 7 The effect of membrane potential variation on ATP stimulation of the ciliary beat frequency. Normalized frequency (*F/Fo*) is plotted as a function of time. ATP stimulation was performed as described in Fig. 5. Each point represents 40 s, subsequently measured and averaged; SE $\leq$ 1%. (*A*) Hyperpolarization of the ciliary cells was achieved by addition of 1 μM amiloride (see Table 1). (*B*) Depolarization of the ciliary cell was achieved by replacing 120 mM Cl^- with SO_4^{2-} (see Table 1).

anions from -28.0 to -8.1 mV (Table 1) and consequently the ciliary frequency initially increased by a factor of ~2 (Bar-Shimon et al., 1992). Addition of extracellular ATP (at the plateau value) further enhanced the ciliary beating (Fig. 7 *B*). Based on these experiments we conclude that the extracellular ATP effect is independent of the initial value of the membrane potential either in the hyperpolarization or depolarization range. It was recently shown that extracellular ATP affects intracellular pH in Ehrlich ascites tumor cells (Wiener et al., 1986) and aortic endothelial cells (Kitazono et al., 1988). In these systems ATP considerably increased the intracellular Na^+ concentration and induced biphasic pH changes. Initially and transiently the cells were acidified; this was followed by sustained alkalization. As was reported, this effect was inhibited by either phorbol esters or amiloride. Such a mechanism is ruled out in our system because amiloride did not inhibit the ATP effect (Fig. 7 *A*).

DISCUSSION

The main goal of this research was to show that extracellular ATP causes hyperpolarization of the membrane potential in

epithelial ciliary cells and to explore its correlation with the increase in beating frequency. ATP-induced membrane hyperpolarization was predicted by Weiss et al. (1992) and verified in this work. The ability of extracellular ATP to hyperpolarize a membrane in a variety of biological systems was recently demonstrated (Gallacher, 1982; Burnstock et al., 1983; Van Coevordeu and Boeynaems, 1984; Kennedy and Burnstock, 1985; Paulmiche et al., 1991; Keef et al., 1992). On the other hand, in other systems, interaction of extracellular ATP with a P_2 receptor resulted in membrane depolarization (Kindmark et al., 1991; Soltoff et al., 1992; Inoue and Brading, 1990). It seems that at this stage of our knowledge, generalizations cannot be made, and each system should be examined separately. Recently, two extracellular ATP binding proteins were identified in the ciliary cells from frog palate and esophagus (Gheber et al., 1992). According to the accepted classification (Dubyak and El-Moatassim, 1993), at least one of them most probably represents a purinoceptor of a P_{2y} subtype. It is generally accepted that stimulations through P_{2y} receptors trigger mobilization of Ca^{2+} via IP_3 pathway (Dubyak and El-Moatassim, 1993). In many cells that possess P_{2y} receptors involvement of calcium-activated potassium channels and membrane hyperpolarization were demonstrated (Gordon, 1986; El-Moatassim et al., 1992).

It is well established that a rise in intracellular Ca^{2+} enhances ciliary motility (Verdugo et al., 1977; Villalon et al., 1989; Hansen et al., 1993; Korngreen and Priel, 1993, 1994). In cultured frog esophagus extracellular ATP induces a rapid, eight-fold increase in intracellular Ca^{2+} concentration. (Fig. 2). Such change in intracellular Ca^{2+} concentration may explain the striking parallelism between enhancement of ciliary beating and membrane hyperpolarization (Figs. 1, 3, and 6) revealed in this work. Depletion of intracellular calcium stores by prolonged exposure to thapsigargin abolished completely both effects (Fig. 3). Moreover, enhancement of ciliary beat frequency and membrane hyperpolarization were also observed after the addition of thapsigargin (Fig. 3) and were probably caused by a thapsigargin-induced rise in intracellular Ca^{2+}. These results strongly indicate that elevation of intracellular Ca^{2+} concentration is sufficient to start a cascade of events that lead to ciliary stimulation and membrane hyperpolarization. The ability of thapsigargin to produce both effects, bypassing the signal transduction pathway from the receptor to the internal stores (Fig. 3), points toward the conclusion that in cultured frog palate and esophagus the main intracellular event triggered by extracellular ATP is Ca^{2+} mobilization from the internal stores.

The extracellular ATP-induced membrane hyperpolarization is achieved only in presence of relatively high concentrations of extracellular Ca^{2+} (Fig. 4 *A*). Extracellular Mg^{2+} can replace Ca^{2+}, although the ATP-induced hyperpolarization in the presence of Mg^{2+} is slightly lower (Fig. 4 *B*). The ability of Mg^{2+} to replace Ca^{2+} in this effect suggests that Ca^{2+} is required as a cofactor. We have also found that potent inhibitors of voltage-gated Ca^{2+} channels, namely verapamil, Ni^{2+}, and Cd^{2+}, in their specific concentrations, do not inhibit the ATP-induced membrane hyperpolarization (data not shown). These results indicate that Ca^{2+} entry is not essential for the ATP-induced membrane hyperpolarization. Similar results were obtained when extracellular ATP-induced ciliary beat frequency enhancement was examined as a function of extracellular Ca^{2+} and Mg^{2+}, and in the presence of potent inhibitors of Ca^{2+} channels (Weiss et al., 1992).

It seems that ATP-induced membrane hyperpolarization plays an important role in ciliary stimulation. Attenuation of the ATP-induced membrane hyperpolarization by Cs^+ (20 mM), quinidine (1 mM), or reduction of the chemical gradient of K^+ across the ciliary cell membrane significantly suppresses the maintenance of ciliary beating enhancement (Figs. 5 and 6, Table 1). Whereas the rapid initial enhancement of ciliary frequency is probably a result of the dramatic increase in intracellular Ca^{2+} (Fig. 2), membrane hyperpolarization is required to maintain a prolonged ciliary beat frequency enhancement, over 10–30 min.

The requirement of intracellular Ca^{2+} rise in order to achieve membrane hyperpolarization indicates possible involvement of calcium-activated potassium channels. The attenuation of ATP-induced membrane hyperpolarization by quinidine (Figs. 5 and 6) supports this suggestion. On the other hand, ATP-induced membrane hyperpolarization is insensitive to charybdotoxin (5 nM), TEA (20 μM), and apamin (1 μM) (Fig. 6). There appear to be three distinct types of K^+ channels activated by Ca^{2+} that differ in their conductance, voltage sensitivity, and susceptibility to specific blockers (Ruff, 1986). One type is blocked by extracellular TEA or by charybdotoxin, another by apamin, and the third by quinidine. Our findings that charybdotoxin, TEA, and apamin had no influence on the ATP effect, and that quinidine is a potent inhibitor, support this classification. There is evidence, however, to show that quinidine is not a specific blocker of Ca^{2+}-activated K^+ channels. For example, in neuroblastoma cells, quinine (an analog of quinidine) blocks both Ca^{2+}-activated and voltage-dependent K^+ channels (DeCoursey et al., 1984). The existence of Ca^{2+}-activated K^+ channels in ciliary systems was shown previously (Brehm and Eckert, 1978; Satow and Kung, 1980; Saimi et al., 1983). Their main physiological role in these systems was believed to be membrane repolarization after depolarization by Ca^{2+} response. It might be possible that in our case these channels cause membrane hyperpolarization as a part of the mechanism of ciliary stimulation by extracellular ATP.

The molecular events induced by membrane hyperpolarization are yet unknown. This effect is independent of the initial membrane potential, since it was observed after initial hyperpolarization or depolarization of the ciliary cells (Fig. 7 and Table 1). In *Paramecium* it was found that stimuli that induce membrane hyperpolarization increase the level of intracellular cAMP (Bonini et al., 1986) and stimulate ciliary activity (Nakaoka and Ooi, 1985; Bonini and Nelson, 1988, 1990). Further work is needed to elicit whether this mechanism may be also applicable in the present case.

Many of the experimental manipulations used in this study had similar effects on the ATP-induced enhancement of ciliary beating and membrane hyperpolarization suggesting that these two parameters are coupled. Moreover, it was shown that membrane hyperpolarization is a necessary condition to achieve prolonged ciliary stimulation. Increased intracellular Ca^{2+}, probably from internal stores, is implicated in the change in membrane potential, most likely by activating calcium-dependent potassium channels. The exact nature of these channels is yet to be determined.

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