Purinergically Induced Membrane Fluidization in Ciliary Cells: Characterization and Control by Calcium and Membrane Potential

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ABSTRACT To examine the role of membrane dynamics in transmembrane signal transduction, we studied changes in membrane fluidity in mucociliary tissues from frog palate and esophagus epithelia stimulated by extracellular ATP. Micromolar concentrations of ATP induced strong changes in fluorescence polarization, possibly indicating membrane fluidization. This effect was dosage dependent, reaching a maximum at 10-μM ATP. It was dependent on the presence of extracellular Ca^{2+} (or Mg^{2+}), though it was insensitive to inhibitors of voltage-gated calcium channels. It was inhibited by thapsigargin and by ionomycin (at low extracellular Ca^{2+} concentration), both of which deplete Ca^{2+} stores. It was inhibited by the calcium-activated potassium channel inhibitors quinidine, charybdotoxin, and apamine and was reduced considerably by replacement of extracellular Na^{+} with K^{+}. Hyperpolarization, or depolarization, of the mucociliary membrane induced membrane fluidization. The degree of membrane fluidization depended on the degree of hyperpolarization or depolarization of the ciliary membrane potential and was considerably lower than the effect induced by extracellular ATP. These results indicate that appreciable membrane fluidization induced by extracellular ATP depends both on an increase in intracellular Ca^{2+}, mainly from its internal stores, and on hyperpolarization of the membrane. Calcium-dependent potassium channels couple the two effects. In light of recent results on the enhancement of ciliary beat frequency, it would appear that extracellular ATP-induced changes both in ciliary beat frequency and in membrane fluidity are triggered by similar signal transduction pathways.

INTRODUCTION

Cilia are projections of the cell surface enveloped by a membrane contiguous with the cell membrane. They are thin (0.25–0.3 μm), relatively long (6–8 μm), and densely packed (100–200 per cell). Cilia beat in an approximately periodic, spatial, and temporal pattern called a metachronal wave. Mucociliary epithelia, whose main function is the transport of material, are found in several organs, including the respiratory tract, the female cervix, and the palate of amphibians. In vivo, in most mucociliary systems, the cilia are usually at rest. They start to beat whenever physiologically needed, triggered by an outside signal. Ciliary cells respond to a variety of stimuli (e.g., mechanical, electrical, hormonal, or neuronal) by altering the pattern of ciliary activity, particularly the frequency (Ovadyahu et al., 1988; Sleigh et al., 1988; Villalon et al., 1989; Sanderson et al., 1990; Aiello et al., 1991; Weiss et al., 1992).

The ciliary membrane appears to play an important role in regulation and modulation of ciliary movement (Eckert, 1972; Naitoh and Kaneko, 1972; Andrivon, 1988). It is well established, for many different membranes, that component lipids and proteins are mobile and undergo rotational Brownian motion in the plane of the membrane (Edidin, 1974; Chapman et al., 1979; Parola, 1993). This mobility (or bilayer fluidity), which is dependent on the tight packing of membrane lipids, is often characterized by a membrane order parameter. This parameter is a function of the mean angle by which the lipid hydrocarbon chain deviates from the normal membrane orientation. Bilayer fluidity is the inverse of bilayer viscosity. Depending on the order parameter, it also reflects other degrees of motional freedom. Membrane dynamics has been shown to play a major role in a variety of physiological processes. There is a growing body of evidence from various cell systems of nonrandom, controlled, and interdependent motions of various membrane components. At the molecular level, the conformation and the corresponding function of membrane channels and receptors, their aggregation state in the membrane, as well as the translational, rotational, and vertical motions of all membrane proteins are controlled by membrane dynamics, specifically lipid–protein interactions (Rimon et al., 1978; Heron et al., 1980; Stubbs and Smith, 1984; Bloodgood, 1992; Parola, 1993). These lipid–protein interactions are probably responsible for fine control of signal transduction steps in the cell membrane.

ATP is known to be an important energy source for many intracellular reactions (including ciliary motility). It was recently found, however, that ATP is released from several cell types, including platelets and purinergic autonomic nerves, and that ATP interacts with purinergic receptors on the surfaces of many cell types (El-Moatassim et al., 1992; Gheber et al., 1995). Because of the great variety of these receptors, a detailed examination of each particular biological system is needed for its characterization.

The pronounced effect of extracellular ATP on ciliary transport rate and beat frequency has been known for a long time (Varhaus and Deyrup, 1953). Recently it was shown that extracellular ATP can enhance the beating of cilia in the trachea of live dogs (Wong and Yeates, 1992). The effect

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initiated by extracellular ATP is one of the strongest produced by any extracellular compound on ciliary beating (Varhaus and Deyrup, 1953; Ovadyahu et al., 1988; Villalon et al., 1989; Weiss et al., 1992; Wong and Yeates, 1992; Gheber and Priel, 1994; Tarasiuk et al., 1995). Given the important role that extracellular ATP may play in stimulating cilia in vivo, we have investigated this system extensively. A detailed examination of the metachronal wave parameters as a function of extracellular ATP concentration was performed (Ovadyahu et al., 1988; Gheber and Priel, 1994). Based on detailed pharmacological data (Weiss et al., 1992) we proposed that the extracellular ATP-induced ciliary excitation is the result of calcium release, mainly from internal stores, and of hyperpolarization of the ciliary membrane. These proposals were recently verified by independent direct methods (Tarasiuk et al., 1995).

The present study was initiated to characterize membrane dynamics during signal transduction and to relate those dynamics to intracellular changes in Ca^{2+}. To the best of our knowledge, membrane dynamics have not been assessed in ciliary cells. In fact relatively little work at all has been performed on whole intact cells of the epithelium using fluorescence probe polarization. Ciliary cells are a uniquely suitable system for such studies. First, they provide a physically observable indicator of cellular signaling (viz., changes in ciliary motility). Second, the components of the axoneme are located near, and essentially wrapped by, the plasma membrane, allowing one to assume that changes in local concentrations of secondary messengers may affect both ciliary beating and membrane dynamics. Extracellular ATP was chosen as a stimulant in our experiments because of its pronounced effect on intracellular processes, as reflected by ciliary motility. We have demonstrated here that, at least for ciliary cells, the extracellular ATP-induced signal transduction cascade produces a remarkable increase in membrane fluidization. It is hoped that this will increase the growing recognition of the role of extracellular ATP and related nucleotides and nucleosides in affecting potent action on cells.

MATERIALS AND METHODS

Materials

4′-Trimethyl-ammonium-1,6-diphenyl-1,3,5-hexatriene (TMA-DPH) and fura-2/AM were obtained from Molecular Probes (Eugene, OR). Ionomycin was obtained from Calbiochem (San Diego, CA). 1,6-diphenyl-1,3,5-hexatriene (DPH), verapamil, amiloride, ATP, AMP-PNP, and all other reagents were from Sigma (St. Louis, MO).

Solutions

All solutions were adjusted to pH 7.2 before use. Two formulations of Ringer's solutions were used. Frog Ringer contained 120-mM NaCl, 2.5-mM KCl, 1.8-mM $CaCl_2$, 1.1-mM Na_2HPO_4, and 0.85-mM NaH_2PO_4. Pig Ringer contained 150-mM NaCl, 2.5-mM Na_2HPO4, 2.5-mM KCl, 1.5-mM $CaCl_2$, 1.5-mM $MgCl_2$, 5-mM D-glucose, and 5-mM HEPES. Solutions with defined Ca^{2+} concentration were prepared by addition of known amounts of Ca^{2+} and 0.5-mM EGTA to Ca^{2+}-free Ringer solution. Free-calcium concentration was calculated as described previously (Jean and Klee, 1986). In all cases in which a stock solution was prepared in an organic solvent (i.e., ionomycin, verapamil, quinidine, and amiloride in ethanol, thapsigargin and Fura-2/AM in DMSO, TMA-DPH and DPH in THF), it was diluted into the experimental solution a thousandfold.

Animals

Experiments were carried out on locally supplied frogs (*Rana ridibunda*) and pigs. Frogs were decapitated and the palates removed. Pig trachea were from pigs killed at a local slaughterhouse. Frog palate and pig trachea were washed and cleaned carefully with the appropriate Ringer solution.

Measurement of fluorescence polarization

Fluorescence polarization is particularly useful for the study of rotational dynamics of a fluorescent probe embedded within the lipid bilayer of a biological membrane (Fuchs et al., 1975; Nathan et al., 1979; Shinitzky and Barenholz, 1978; Parola, 1993). On illumination of the labeled tissue with polarized light, polarized emission is detected. The extent to which the emitted light is polarized depends primarily on the angle between the absorption and emission dipole moments of the probe, its rotational Brownian motion during the excited state lifetime, and its fluorescence lifetime. The magnitude of rotational Brownian motion depends on the size and shape of the probe molecule, its surrounding microviscosity, and the temperature (Shinitzky and Barenholz, 1978). When fluorescence polarization measurements are obtained at constant temperature and probe lifetime, they reflect the probe's rotational mobility, which permits the estimation of the microviscosity of the membrane surroundings. Front-face fluorescence measurements were carried out with an MPF-44 Perkin Elmer spectrofluorimeter and calculated as described previously (Nathan et al., 1979). This method is a very reliable substitute for absolute measurement for the determination of relative changes in fluorescence anisotropy (Eisinger and Flores, 1979). The labeled tissue was excited with light at 360 nm. The emission was measured at 430 nm after filtration with a 400-nm cutoff filter as described previously (Nathan et al., 1979). Briefly, fluorescence polarization (P) was calculated from the equation

$$P = (I_{0,0} + GI_{0,90})/(I_{0,0} - GI_{0,90}),$$

where $I_{0,0}$ and $I_{0,90}$ represent the intensity, corrected for unlabeled blank tissue contribution ($\leq 5\%$), of the polarized emission parallel and normal to the vertically polarized excitation beam. A correction factor ($G = I_{90,0}/I_{90,90}$) was used to correct for unequal transmission of polarized light by the instrument.

Preparations and procedure

For fluorescence measurements an inclined insert made of black PVC was placed inside a 1 × 1 cm quartz cuvette. The slope of this insert was in the (1,1,1) plane, by analogy with the crystallographic plane in cubic crystals. On top of this plane the freshly excised frog palate or pig trachea was placed, tied with a black thread and immersed in Ringer's solution. The tissue was labeled with 2×10^{-5} M TMA-DPH or 2×10^{-6} M DPH according to published procedures (Nathan et al., 1979; Parola et al., 1981). The 10-fold difference in concentration between the DPH and the TMA-DPH was due to the more favorable partition of DPH between water and lipid phases. Charged TMA-DPH partially dissolves in the aqueous phase in which it is nonfluorescent. This was reexamined and specifically verified in our system. Each measurement was repeated at least five times, and the mean fluorescence polarization values were reported. For each agent studied at any given concentration, freshly excised frog palates from 5 to 10 animals were used. In control experiments, an equivalent amount of Ringer's solution alone was substituted for the drug or reagent. Under these conditions, no changes were observed in the fluorescence polarization values (P). At the end of each experiment the cilia were still beating

normally, indicating unaltered vitality. The value of the fluorescence polarization of the tissue sample was characterized as follows: i) labeling conditions were reexamined and determined to be similar to those described before (Parola et al., 1981), ii) P was determined not to be dependent on slit width, indicating the homogeneity of the measured sample, and iii) setting the tissue in a geometry similar to that already reported in other systems (Toplak et al., 1990) resulted in the same P values as obtained in our geometry. This indicated that scattered and reflected light did not adversely affect the measurements.

Fluorescence-lifetime measurements

An SLM-4800 spectrofluorimeter, modified by ISS Inc. (Champaign, IL) to a GREGG-MM multifrequency phase modulation spectrofluorimeter, was used for measuring lifetimes at room temperature, with the sample holder configured for front-face geometry as done for steady-state fluorescence polarization measurements. To enhance intensity and lower the spurious effect of dynamic polarization, a depolarizer was inserted in the excitation beam. Emission was detected through a polarizer tilted 36° from the vertical. The excitation source was a 450-W Xe lamp, tuned to λ_{ex} = 360 nm (slit 4/4). A KNO_2 liquid filter was used for emission. The modulation frequency range was 10–100 MHz. The reference sample was POPOP (τ = 1.35 ns). Fluorescence decay analysis was applied, utilizing the ISS global analysis. Lifetime measurements were taken before and immediately after the addition of ATP. The lifetime measurement lasted ~30 min and overlapped the effect of extracellular ATP. In the lifetime analysis program, SE values of 0.004 for modulation and 0.200 for the phase angle were chosen. The choice of a two-lifetime analysis model yielded the best χ^2 value.

Intracellular calcium measurements

We measured the intracellular free calcium concentration ($[Ca^{2+}]_i$) in a single ciliary cell grown in culture, using the fluorescence indicator fura-2 as previously described (Korngreen and Priel, 1994). The calcium concentration was measured from a single ciliary cell in culture and not from the excised palate because unspecified loading of nonciliated cells with fura-2 and the high autofluorescence displayed by the excised palate might cause severe inaccuracies in the measurement. The beat frequency of the cilia in the excised palate and in the tissue culture was monitored at rest, and after the addition of extracellular ATP, by the photoelectric method (Eshel et al., 1985). No difference was observed between the characteristics of the ciliary beat frequency in the excised palate and in tissue culture, as has also been observed by others (Welsh, 1987; Kennedy and Ranyard, 1983). Cells were loaded with 5 μM of fura-2/AM in 1 ml of growth medium for 30 min at room temperature and washed three times with Ringer's solution. The cells were epi-illuminated with light alternately at 340 and 380 nm. The 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 were stored in a computer at intervals of 2 s and averaged to give one data point every 40 s. Inasmuch as we encountered a high failure rate when loading fura-2 into the cells, measurements were carried out only on cells that displayed a high fluorescence intensity and normal ciliary beating.

At relatively high concentration (1 mM), extracellular ATP can increase nonselective permeability of the cell membrane. This was observed primarily in transformed cells (El-Moatassim et al., 1992). Such a permeabilization phenomenon could increase membrane fluidity on its own. To rule out such a possibility we examined whether 1-mM extracellular ATP permits the entrance of fura-2 potassium salt (to which the membrane is not permeable) into the cells. The failure to load the anionic form of fura-2 into these cells indicated that extracellular ATP, even at this high concentration, does not cause membrane permeabilization in the discussed system.

RESULTS

The average value of the fluorescence polarization (P) of the ciliary membrane from frog palate was 0.178 ± 0.002. This average was performed over a relatively large number of animals (n = 201). Because of the absence of fluorescence polarization measurements from ciliary cells, it is impossible to compare this value with literature values. However, it is quite consistent with our measurements for ciliated cells from frog esophagus and pig trachea. It is lower, however, than P values measured in nonciliated cells. In some ciliary systems, membrane fluidities and lipid composition differ between the cilium and plasma membranes (Thompson and Nozawa 1977; Andrews and Nelson 1979). The questions of what makes the ciliary membrane more fluid and why are beyond the scope of this work. Our interest was primarily in the relative (normalized) changes in P values induced by the agonists tested. It was observed that the relative change in P in numerous experiments on different samples of frog palate and pig trachea was quite constant, implying that the same percent change was observed regardless of the initial P_0 value. Therefore each experiment was normalized to the average value of the fluorescence polarization before the addition of the agonist (P_0). These average, normalized results are presented in Table 1.

Effect of extracellular ATP on membrane fluidity

Addition of extracellular ATP to ciliary epithelia from frog palate (Fig. 1 *A*), labeled with either TMA-DPH or DPH, in Ringer's solution has a profound effect on fluorescence

TABLE 1 Effect of purinergic stimulation on the maximal change in fluorescence polarization

Treatment	Pretreatment*	P_{max}/P_0 (%)‡
5-μM ATP	–	5 ± 1.0 (6)
10-μM ATP	–	20 ± 0.7 (20)
50-μM ATP	–	6 ± 0.8 (7)
10-μM AMP-PNP	–	16 ± 0.9 (5)
10-μM ATP	1-μM thapsigargin§	2 ± 0.6 (6)
10-μM ATP	5-μM ionomycin§	4 ± 1.2 (5)
10-μM ATP	10-μM verapamil	20 ± 0.9 (5)
10-μM ATP	5-mM Ni^{2+}	18 ± 1.2 (5)
10-μM ATP	0.2-mM quinidine	2 ± 0.4 (7)
10-μM ATP	20-nM charybdotoxin	5 ± 0.9 (5)
10-μM ATP	0.5-μM apamine	4 ± 1.1 (5)
10-μM ATP	5-mM Cs^+	6 ± 0.7 (5)

TMA-DPH-labeled frog palates were treated with ATP or its nonhydrolyzable analog AMP-PNP. The effect of ATP was examined after the depletion of the internal calcium stores with thapsigargin and ionomycin after the application of the calcium channel inhibitors verapamil and Ni^{2+} and after the addition of the potassium channels blockers quinidine, charybdotoxin, apamine, and Cs^+. The maximal change (P_{max}) of the fluorescence polarization is normalized to the control value (P_0) and displayed as percent.

*Incubation time 30 min.

‡Mean ± SE (n).

§$[Ca^{2+}]_0 < 1$ μM, $[Mg^{2+}]_0$ = 1.8 mM, incubation time 90 min.

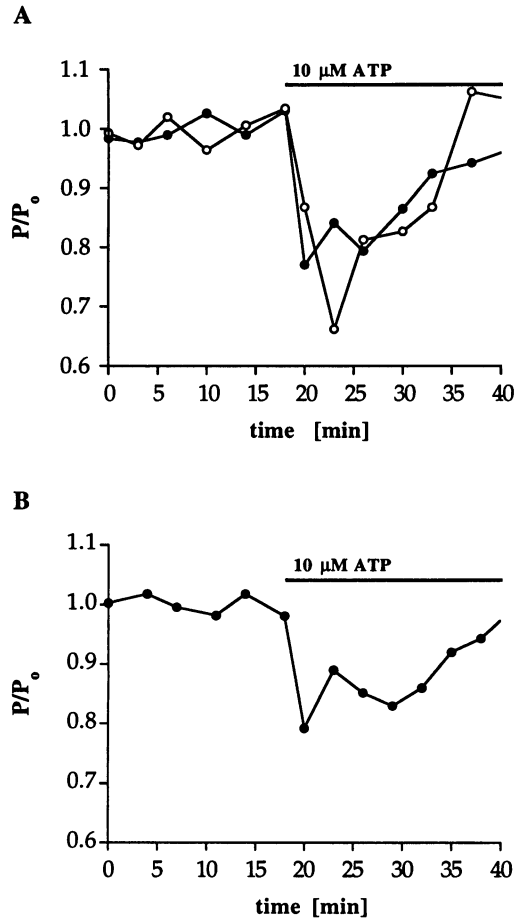

FIGURE 1 Effect of extracellular ATP on fluorescence polarization. (*A*) Effect of 10-μM extracellular ATP on the normalized fluorescence polarization of DPH- (○) and TMA-DPH- (●) labeled frog palates. (*B*) Effect of 10-μM extracellular ATP on the normalized fluorescence polarization of TMA-DPH-labeled pig trachea. Exposure to light was for less than 5 s on each reading; the G factor was measured each time.

polarization. The response to addition of extracellular ATP was fast, a minute or less, followed by relatively slow recovery. The recovery time when the culture was labeled with TMA-DPH was 15–30 min. Significant differences between the two probes were observed. Although TMA-DPH (20 μM) revealed an average decrease in fluorescence polarization of 20 ± 3%, DPH (2 μM) showed an average reduction of 35 ± 5% in response to extracellular ATP. The more pronounced effect with DPH could be due to its localization in the depth of the lipid core of the cell membrane, which would be nonspecific and time dependent. Therefore, all the measurements described below were performed with TMA-DPH, whose location is well defined, anchored to the phospholipid head groups at the lipid–water interface. Similar effects were observed with pig trachea (Fig. 1 *B*) as well as with frog esophagus (data not shown). It seems that the membrane fluidization induced by extracellular ATP is not limited to frog palate epithelium but is quite general, at least in mucociliary systems. In all the tissues examined, the pronounced change in polarization obtained after the addition of extracellular ATP is not associated with a change in fluorescence lifetime as measured by multifrequency phase modulation spectrofluorimetry (Fig. 2). Lifetime analysis (Gratton et al., 1984; Parola et al., 1990) resulted in τ_1 = 6.06 ns, with a major fractional contribution of 0.95, and τ_2 = 2.54 ns, with a quite negligible fractional contribution of 0.05 (χ^2 = 0.905), indicating that the change in fluorescence polarization resulted mainly from changes in membrane fluidity. It should be emphasized that changes in fluorescence polarization of such magnitude indicate that pronounced changes in membrane viscosity have occurred. For example, according to the calibration curve for DPH of Shinitzky and Barenholz (1978), a change of 35% in fluorescence polarization value indicates a change of 42% in the viscosity of the probe environment. Such strong effects are rarely observed in viable cells.

Membrane fluidization induced by extracellular ATP changed drastically in the 5–50-μM range (Table 1). Increasing ATP concentration from 5 to 10 μM increased membrane fluidity, as has been demonstrated for ciliary beat frequency (Weiss et al., 1992). Higher extracellular ATP

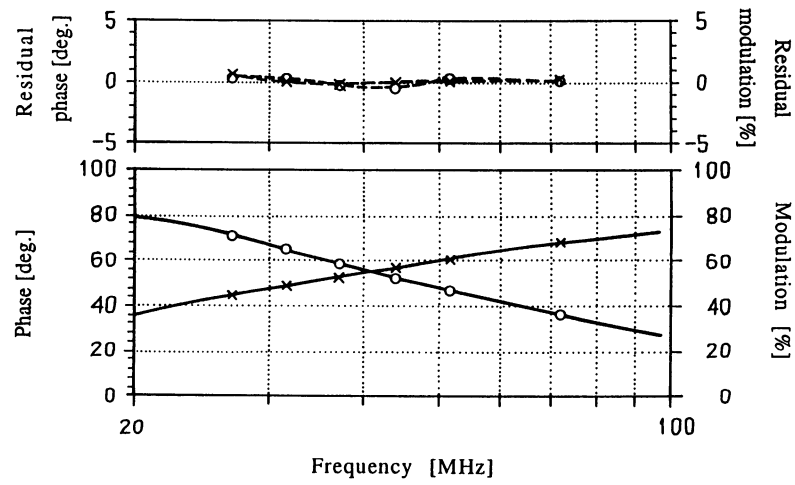

FIGURE 2 Fluorescence lifetime measurement of freshly isolated frog palate stained with TMA-DPH, to which 10-μM ATP has been added. The modulation (○) and the phase (×) are presented as a function of frequency. The residual phase and modulation are presented in the top trace. The two lifetimes were τ_1 = 6.06 ns and τ_2 = 2.54 ns; χ^2 = 0.905. The addition of ATP did not change the lifetime; hence the change in fluorescence polarization was assigned to membrane fluidity.

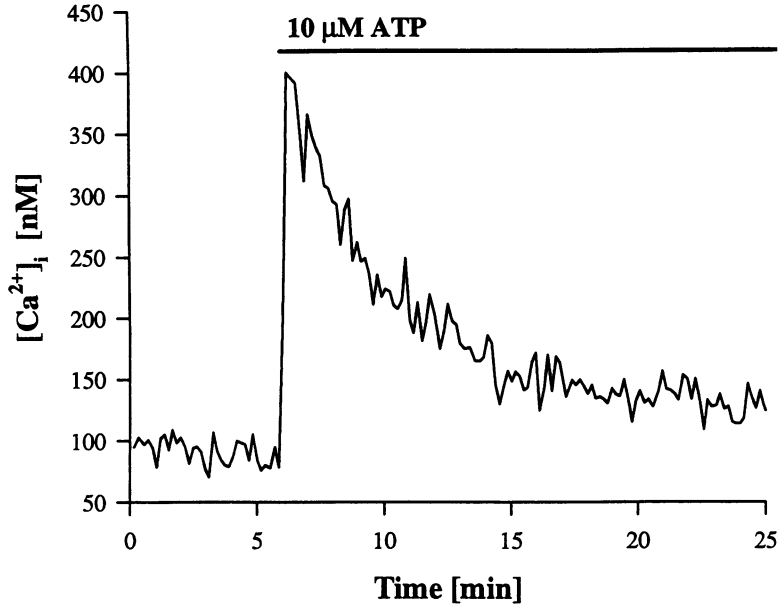

FIGURE 3 Effect of 10 μM of extracellular ATP on the intracellular free-calcium concentration. Experiments were carried out on monolayer cultures from frog palate epithelium loaded with 5-mM fura-2/AM. The final concentration of DMSO in the loading medium was less than 0.1% (v/v). Similar results were obtained in five other experiments.

concentration caused a drop in membrane fluidity. The dependence of membrane fluidization on extracellular ATP concentration very much resembles the dose-dependent behavior of the ciliary beat frequency response to extracellular ATP (Weiss et al., 1992). The ATP induced fluidization was not due to hydrolysis of the ATP because similar effects were obtained with AMP-PNP, a nonhydrolyzable analog of ATP (Table 1). Therefore, ATP must be exerting its extracellular stimulatory effect by interaction with its membrane purinoceptor and not by its use as an energy source, as was recently demonstrated in our experimental system (Gheber et al., 1995).

Involvement of Ca^{2+} in membrane fluidization by ATP

Fig. 3 demonstrates the time-dependent behavior of $[Ca^{2+}]_i$ induced by extracellular ATP. As can be seen, administration of 10-μM extracellular ATP caused a fourfold rise in intracellular Ca^{2+} as measured by fura-2 fluorescence. The response to ATP stimulation was fast (several tens of seconds), decaying slowly over 20 min.

Similar results were recently obtained in ciliary cells from rabbit oviduct (Villalon et al., 1989) and rabbit trachea (Korngreen and Priel, 1994) and in human nasal polyps (Korngreen and Priel, 1993). On the basis of these findings, the roles of intracellular and extracellular Ca^{2+} and voltage-dependent calcium channels in the process of membrane fluidization by ATP were examined more extensively. Fig. 4 depicts the effect of ionomycin, a highly potent ionophore for Ca^{2+}, on fluorescence polarization. The addition of 2 μM of ionomycin increased the intracellular Ca^{2+} concentration (at 1.8-mM extracellular calcium concentration) and increased the fluidity of the ciliary membrane. However, the increase in membrane fluidity owing to increased intracellular Ca^{2+} by ionomycin was less pronounced than the effect observed with extracellular ATP, 12 ± 1% versus 20 ± 3%, respectively (Fig. 1 *A* and Fig. 4). According to our previous results (Korngreen and Priel, 1993, 1994) the rise of intracellular Ca^{2+} induced either by ionomycin or by extracellular ATP was of approximately the same value. Increasing ionomycin concentration from 2 to 7 μM did not increase the magnitude of the observed effect (Fig. 4), indicating that this reagent had achieved its maximum effect. The relationship among extracellular ATP, intracellu-

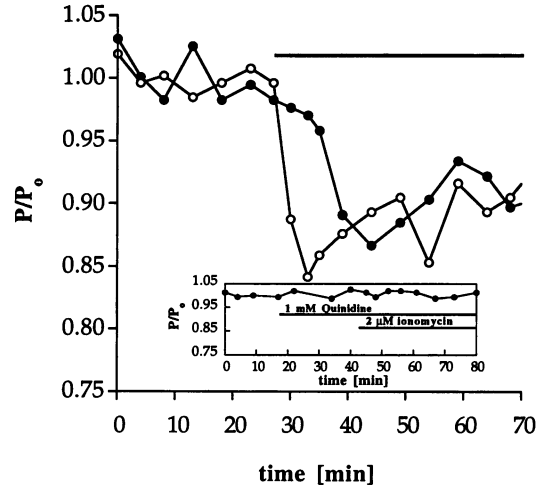

FIGURE 4 Effect of ionomycin on fluorescence polarization. Ionomycin, at 2 μM (●) and 7 μM (○), causes a drop in fluorescence polarization. The horizontal bar indicates the addition of the agonist. Addition of 0.2-mM quinidine (*inset*), a Ca^{2+}-dependent K^+ channel blocker, completely inhibits the effect of 2-μM ionomycin. Both ionomycin and quinidine were dissolved in ethanol and diluted in Ringer's solution to the desired concentration. The final concentration of ethanol was less than 0.1%.

lar Ca^{2+}, and membrane fluidity is, therefore, not a straightforward one.

These results indicate that a rise in intracellular Ca^{2+} concentration is involved in the process of membrane fluidization. Extracellular ATP may elicit such a rise either by mobilization of internal stores of Ca^{2+} or by increased influx of external Ca^{2+} into the cell through control of voltage-gated Ca^{2+} channels (Villalon et al., 1989). To test for the involvement of internal calcium stores, we depleted those stores with thapsigargin, a well-known membrane permeable inhibitor of the endoplasmic reticulum's calcium ATPase (Lytton et al., 1991). Incubation of ciliary tissue from frog palate for 90 min with 1-μM thapsigargin (in Ringer's solution with 0.1-μM calcium and 1.8-mM $MgCl_2$) completely abolished the ATP induced membrane fluidization (Table 1). We obtained similar results (Table 1) by depleting calcium internal stores with 5-μM ionomycin, at 0.1-μM extracellular calcium, for 90 min. This provides strong evidence that extracellular ATP elicits its effect on membrane fluidity through a mechanism involving release of calcium from internal stores.

To test for the involvement of external Ca^{2+}, membrane fluidization by extracellular ATP was examined over a wide range of exogenous Ca^{2+} concentrations. Figure 5 clearly shows that ATP-induced membrane fluidization is a function of extracellular calcium concentration. The TMA-DPH fluorescence polarization values displayed a gradual decrease from 1 to 100 μM $[Ca^{2+}]_0$, followed by a sharper decrease from 100 μM to 1.8 mM $[Ca^{2+}]_0$. Our experiments were thereafter carried out at 1.8-mM extracellular Ca^{2+} concentration, which is the normal Ringer concentration. To check the specificity of the ATP effect for calcium, we replaced the exogenous calcium by magnesium. The free Ca^{2+} concentration in this experiment was quite low (1 μM), a concentration already shown in Fig. 5 to have a small effect on the fluorescence polarization. It was found that, in these experiments, magnesium could replace calcium and produce an effect essentially the same as that of Ca^{2+} (data not shown). Therefore, in the experiments with thapsigargin, magnesium was substituted for calcium in the solution.

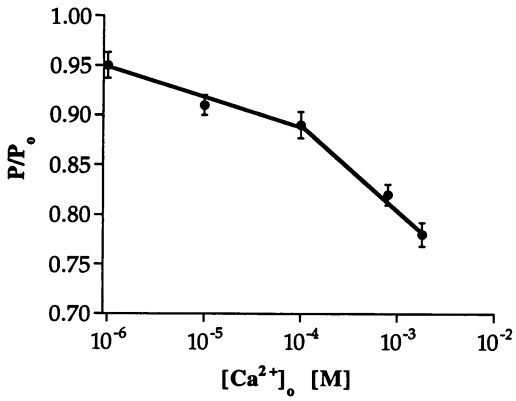

FIGURE 5 Effect of the concentration of extracellular calcium on the ATP-induced fluorescence polarization. The excised frog palate was washed several times in Ringer's solution containing the desired concentration of extracellular calcium to remove completely all traces of calcium from the palate. The palate was incubated in the experimental solution for 30 min before the experiment. Fluorescence polarization was monitored for 20 min, and 10-μM extracellular ATP was added after this period. Results are presented as mean ± SE (n = 5).

Recently it was found in human nasal polyps (Korngreen and Priel, 1993) that the ATP-induced elevation of intracellular Ca^{2+} is biphasic as a function of extracellular Ca^{2+}. From 0.1- to 10-μM extracellular Ca^{2+} the extracellular ATP-induced rise of intracellular Ca^{2+} was constant. However, above 10-μM extracellular Ca^{2+} the ATP-induced intracellular Ca^{2+} release increased as a function of extracellular Ca^{2+} concentration. Interestingly, although this is from a different system, these findings correlate with the ATP-induced membrane fluidization dependence on extracellular Ca^{2+} that we also observed, as shown in Fig. 5.

To test whether ATP-induced membrane fluidization was also affected by voltage-gated Ca^{2+} channels, verapamil, a potent blocker of voltage gated Ca^{2+} channels, was employed. Addition of 10-μM verapamil (the upper concentration limit of its specificity) influenced neither the ATP effect nor the initial value of membrane fluorescence polarization using TMA-DPH as the probe (Table 1). Moreover, experiments carried out in the presence of up to 5-mM Ni^{2+} (a known voltage-gated calcium channel blocker at a concentration well below 5 mM), failed to detect any change in the ATP response (Table 1). It seems, therefore, that although ATP-induced membrane fluidization is affected by external Ca^{2+} concentration, that effect is not mediated through voltage-gated calcium channels.

Involvement of K^+ channels in the effect of exogenous ATP

Quinidine, a potent inhibitor of calcium-dependent potassium channels, abolished the effect of extracellular ATP on membrane fluidity (Table 1). The concentration of quinidine used in these experiments was 0.2 mM, and that of ATP was 10 μM, a concentration at which maximal effect was achieved. Addition of quinidine alone (without ATP) did not influence membrane fluidity. Moreover, 0.2-mM quinidine completely blocked the ionomycin-induced membrane fluidization (Fig. 4, *inset*). These results suggest a possible involvement of calcium-activated potassium channels in the process of ATP- and ionomycin-induced membrane fluidization. Similarly, charybdotoxin and apamine, additional blockers of calcium-dependent potassium channels, at 20 nM and 0.5 μM, respectively, and Cs^+, a nonspecific blocker of potassium channels at 5 mM, partially inhibited the extracellular ATP effect (Table 1). Control experiments in the absence of extracellular ATP showed that none of the channel blockers affected the membrane fluidity as measured by fluorescence polarization (data not shown).

To investigate the influence of K^+ flux through the cell membrane on the fluidization effect by ATP, we gradually

raised the extracellular concentration of K^+ from 2.5 to 122.5 mM. To keep the osmolarity constant, Na^+ concentration in the Ringer solution was lowered, proportionate to the increase K^+ concentration. Figure 6 demonstrates that raising the external K^+ concentration from 2.5 to 30 mM considerably lowered membrane fluidization induced by 10-μM ATP. Further increase in extracellular K^+ concentration (from 30 to 122.5 mM) continued to lower the ATP-induced membrane fluidization, though at a more moderate rate. Replacement of extracellular Na^+ ions by K^+ causes a concentration-dependent depolarization of ciliary membrane, which, in turn, increases the fluidity of the ciliary membrane. Therefore, the 10-μM extracellular ATP was applied only after 30-min preincubation with the test solution to normalize the fluorescence polarization value for the depolarized membrane. The net ATP effect under the given conditions was therefore assessed to exclude effects arising from membrane depolarization. On the basis of these findings it is possible to conclude that calcium-activated potassium channels play an important role in membrane fluidization by extracellular ATP.

Dependence of membrane fluidization on membrane potential

Involvement of calcium-activated potassium channels should lead to hyperpolarization of ciliary membrane. Indeed, it was shown recently that extracellular ATP induces hyperpolarization in the system under study (Tarasiuk et al., 1995). The hyperpolarization is coupled to the rise of $[Ca^{2+}]_i$ through the existence of these Ca^{2+}-activated K^+ channels. Therefore, to assess the net effect of membrane potential on membrane fluidization, we induced both hyperpolarization and depolarization in the ciliary membranes.

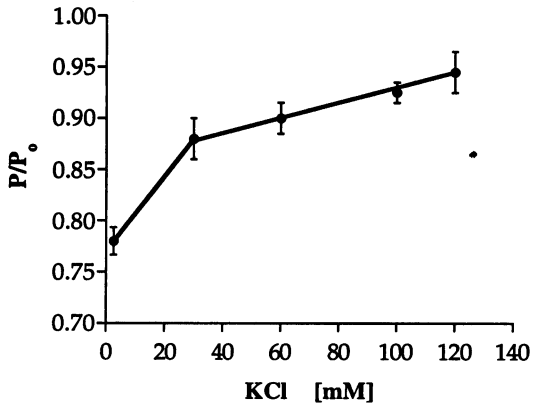

FIGURE 6 Effect of the plasma membrane K^+ gradient on the ATP-induced fluorescence polarization. The plasma membrane was depolarized by replacement of the sodium ions in the Ringer solution with potassium ions. The excised frog palate was washed several times with Ringer's solution containing the desired concentration of KCl and incubated in this solution for 30 min before the experiment. The fluorescence polarization was monitored for 20 min, and 10-μM extracellular ATP was added after this period. Results are presented as mean ± SE (n = 5).

Amiloride, a potent blocker of Na^+ channels, induced strong hyperpolarization (30%) of the membrane. As can be seen from Fig. 7 *A*, addition of 1-μM amiloride caused a prolonged fluidization of the ciliary membrane. However, the maximal effect of membrane fluidization caused by amiloride is half that of the ATP effect. In addition, experiments were performed in Ringer's solution in which equivalent amounts of extracellular Na^+ were replaced by *N*-methyl D-glucamine. Lowering the Na^+ concentration increased linearly the degree of hyperpolarization.

Hyperpolarization of the ciliary membrane by altering Na^+ concentration induced a dose-dependent fluidization of the membrane (Fig. 7 *B*). Lowering the extracellular Na^+ concentration from 123 to 30 mM induced an almost linear decrease of normalized fluorescence polarization; further decrease of $[Na^+]_{out}$ from 30 to 15 mM resulted in a sharp drop of P_{max}/P_0, reaching asymptotically its minimum value. This behavior may indicate a possible phase transition of membrane organization. Although this is not directly related to the scope of this work, depolarization of the ciliary membrane by addition of 5-mM Ba^{2+} to the Ringer solution or by dose-dependent replacement of $[Na^+]_{out}$ by $[K^+]_{out}$ resulted in similar changes in P (data not shown).

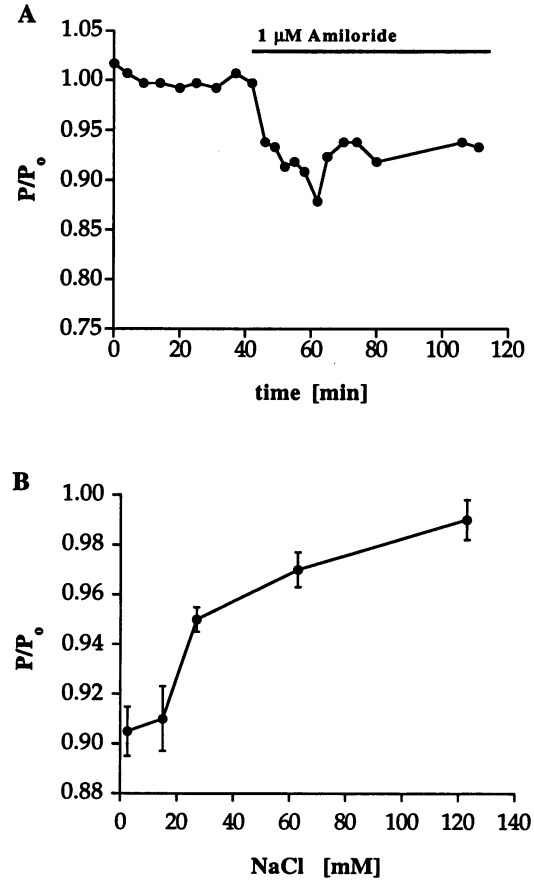

FIGURE 7 Effect of plasma membrane hyperpolarization on fluorescence polarization. (*A*) The plasma membrane was hyperpolarized by the addition of 1 μM of amiloride and (*B*) by replacement of the sodium ion in the Ringer solution with *N*-methyl D-glucamine. The results are presented as mean ± SE (n = 5).

In summary, hyperpolarization or depolarization of ciliary membrane induces membrane fluidization. However, the effect induced by ATP is considerably different in its time course and maximal magnitude from the effects observed with hyperpolarization or depolarization. Whereas the ATP effect is transient, recovering slowly to its normal value, the change of membrane fluidity as the result of hyperpolarization or depolarization is nearly constant and recovers only by a change in the membrane potential to its resting value. The magnitude of the maximal effect is also considerably smaller. The maximal effect achieved by strong hyperpolarization or depolarization is half the maximal effect achieved by ATP. According to our measurements, extracellular ATP induces a change of membrane potential of 25%, whereas amiloride and removal of Na^+ induce hyperpolarization of 30% and 40%, respectively (unpublished data). This might indicate that membrane fluidization achieved by ATP may be correlated with at least two coupled events, for example, the rise of $[Ca^{2+}]_i$ and hyperpolarization.

DISCUSSION

The ability of extracellular ATP to induce considerable membrane fluidization as an integral part of signal transduction has been presented here for the first time. The effect appears to be quite general, at least for mucociliary systems; a similar effect is observed in amphibian (frog esophagus and palate, Fig. 1 *A*) and in mammal (pig trachea, Fig. 1 *B*). It is reasonable to hypothesize that the induced membrane fluidization by extracellular ATP revealed in this work is an outcome of the intracellular events imposed by extracellular ATP. A major purpose of this work was to cast light on this hypothesis. It is well known that extracellular ATP may induce mobilization of Ca^{2+} primarily from internal stores via the inositol phosphate pathway (Dubyak and El-Moatassim, 1993). Indeed, it was shown that ATP causes up to a fourfold increase in intracellular calcium concentration (Fig. 3). Increasing the intracellular calcium concentration by ionomycin, at normal extracellular calcium concentration, induced membrane fluidization (Fig. 4). Moreover, depletion of intracellular Ca^{2+} stores, by either thapsigargin or ionomycin at low extracellular Ca^{2+} concentration (10^{-7} M) and in the presence of 1.8-mM Mg^{2+}, completely abolished the membrane fluidization by exogenous ATP. These results strongly indicate that the rise of intracellular Ca^{2+} is a necessary condition for achieving membrane fluidization by extracellular ATP. It was demonstrated by using organic (verapamil) and inorganic (Ni^{2+}) inhibitors that voltage-gated calcium channels are not involved in the process. On the other hand, the induced membrane fluidization by extracellular ATP depended on extracellular free-Ca^{2+} concentration (Fig. 5). In addition, extracellular Ca^{2+} could be replaced by Mg^{2+}, achieving a similar effect of membrane fluidization by ATP. These results support the argument that exogenous Ca^{2+} is involved in ATP effects but most probably not by entering the cell through a voltage-gated Ca^{2+} channel.

The dependence of the ATP effect on quinidine, charybdotoxin, apamine, Cs^+, and extracellular K^+ concentration (Table 1) indicates the involvement of calcium-dependent potassium channels. Recently it was shown (Tarasiuk et al., 1995) that opening of these channels leads to hyperpolarization of the ciliary membrane from frog esophagus and palate. It has been suggested that hyperpolarization induces a rise in intracellular cAMP concentration (Bonini and Nelson, 1988). An alternative explanation that leads to the same conclusion is that K^+ efflux through channels that carry relatively strong fluxes appears to serve a nonelectrical function, namely, the direct control of cAMP formation (Schultz et al., 1992). In any event, hyperpolarization of the ciliary membrane, probably by K^+ (Ca^{2+}) channels, plays an important role in the ATP-induced membrane fluidization. Inhibition of these channels by their specific blockers reduced the ATP effect considerably (Table 1) and completely abolished the ionomycin effect (Fig. 4, inset). Furthermore, reducing the cross-membrane gradient of K^+ also reduced the ATP effect (Fig. 6). Hyperpolarization of the ciliary cell without activating calcium-dependent potassium channels also induced membrane fluidization (Fig. 7). However, this effect was smaller than the ATP effect. These results lead us to the conclusion that an enhancement of intracellular Ca^{2+} is a necessary, but insufficient, condition for achieving remarkable ($\geq$20%) membrane fluidization. In addition to the intracellular Ca^{2+} increase, membrane hyperpolarization is probably needed. Calcium-dependent potassium channels couple between these effects.

On the basis of our findings, the following mechanism may be suggested: extracellular ATP interacts with a purinoceptor; this interaction starts a cascade of events, ending in increased intracellular free-Ca^{2+} concentration, released mainly 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, which will lead to membrane hyperpolarization, and ii) increased intracellular (most probably near the membrane) free-Ca^{2+} concentration and membrane hyperpolarization induces changes in membrane organization, leading to appreciable membrane fluidization.

Qualitatively, all our results confirm the suggested model. However, according to the model, ionomycin (at normal extracellular calcium concentrations) is supposed to induce an equal effect to that of ATP. According to our model the calcium-activated potassium channels should be opened, resulting in membrane fluidization. Ionomycin, however, induced half the fluidization of ATP (compare Figs. 1 *A* and 4). Moreover, increasing the ionomycin concentration from 2 to 7 μM did not change the magnitude of the effect, indicating that it is the maximal effect achievable by ionomycin. This discrepancy leads us to the conclusion that the suggested model is oversimplified. It seems that the extracellular ATP pathway activates additional effectors,

which contribute to the strong and sustained membrane fluidization. This further enhances the observation that the extracellular ATP-induced membrane fluidization is an integral part of the signal transduction pathways leading to ciliary stimulation. To the best of our knowledge this is the first time that this effect, which may be one of distinct importance to other cell types, has been observed.

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