Functional cross talk after activation of P2 and P1 receptors in oviductal ciliated cells

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Received 24 September 1998; accepted in final form 16 March 2000

Mora, Bernardo, Nelson Barrera, Pablo Uribe, Claudio Mora, and Manuel Villalón. Functional cross talk after activation of P2 and P1 receptors in oviductal ciliated cells. Am J Physiol Cell Physiol 279: C658–C669, 2000.—The presence of ATP and adenosine receptors and their role in controlling ciliary activity in oviductal ciliated cells was studied by measuring the ciliary beat frequency (CBF) in oviductal tissue cultures. ATP, adenosine, and related compounds increased the CBF in a dose-dependent manner. We established that P2 receptors of subtype 2Y2 and P1 receptors of subtype A2a mediated the responses to ATP and adenosine, respectively. We found evidence to suggest that stimulation of ciliary activity by ATP requires d-myo-inositol 1,4,5-trisphosphate [Ins(1,4,5)P3] metabolism, intracellular Ca2+ mobilization, and protein kinase C activation. On the other hand, the adenosine effect is mediated by activation of a Gs protein-dependent pathway that enhances cAMP intracellular levels. To study the interaction between P2 and P1 receptors, cells were stimulated simultaneously with both agonists. We observed a synergistic increase of the CBF even at agonist concentrations (100 nM) that did not produce a significant response when added separately to the culture. Furthermore, a blocker of the cAMP pathway produced a reduction of the ATP response, whereas a blocker of the Ins(1,4,5)P3 pathway also produced an inhibition of the adenosine response. Our evidence demonstrates that both ATP and adenosine receptors are present in a single ciliated cell and that a mechanism of cross talk could operate in the transduction pathways to control ciliary activity.

Ciliated cells modify their ciliary beat frequency (CBF) in response to a variety of mechanical, electrical, and chemical stimuli. These signals are involved in the control of mucociliary transport velocity, which plays a fundamental role in the “clearance” of several types of epithelium (47). In oviductal epithelium, ciliary activity is also important in the transport of gametes and embryos through the oviduct (23), and there is evidence that chemical signals, including sexual hormones, control CBF (47).

The effect of extracellular ATP on ciliary transport and beat frequency has been well established in a variety of ciliated cells (10, 26, 45). Micromolar concentrations of ATP are able to induce an increase in the CBF of ciliated cells of frog palate and esophagus epithelium (15, 40, 49), salamander and rabbit oviductal epithelium (26, 45), and rat and human airway epithelium (10, 33). These effects are believed to be mediated by a diverse family of membrane receptors, termed P2 receptors (12). Recent receptor nomenclature based on molecular and functional characterization provides evidence for two extended families of ionotropic (P2X12Y2) and metabotropic (P2Y1n) receptors activated by either purine or pyrimidine nucleotides, or both (16). Extracellular nucleotides are thought to be involved in a number of physiological functions that are initiated by the interaction of ATP with membrane receptors, a reaction that triggers a sequence of intracellular biochemical events (8). Ciliated cells from frog palate and esophagus epithelia increase their CBF after exposure to ATP through a mechanism involving membrane receptor activation and mobilization of intracellular Ca2+ from internal stores (49). Furthermore, an increase in cytosolic free Ca2+ opens a Ca2+-activated K+ channel, leading to membrane hyperpolarization, which enhances CBF by a mechanism that is as yet unknown (40). We demonstrated previously that the ATP-dependent increase of CBF in ciliated cells of oviductal epithelium was associated with a rise in cytoplasmic Ca2+ (45). However, there are no reports that characterize nucleotide receptors and the molecular events underlying ATP-induced stimulation of ciliary activity.

Previous studies have demonstrated that adenosine can modulate ciliary activity (39, 49) through its interaction with P1 membrane receptors, of which there are four types (9, 12, 43). The A1 subtype, with a high affinity for the agonist, inhibits adenylyl cyclase activity; the A2 subtypes, with a low agonist affinity but which stimulate adenylyl cyclase, are termed A2a and A2b (12, 37). The A3 receptor subtype, present in the cardiovascular system (36), is like the A1 subtype in
that it inhibits adenylyl cyclase activity (11). It is known that cAMP increases the CBF in rabbit tracheal (19, 38, 39), amphibian oviduct (26), canine airway (50), and human respiratory epithelium (7). However, Tamaoki et al. (38, 39) found evidence for an adenosine-mediated, cAMP-dependent inhibition of ciliary activity in rabbit tracheal epithelium cultures, whereas adenosine enhances ciliary activity in monolayer tissue cultures grown from excised frog palate and esophagus epithelium (49). Although the action of adenosine seems to be through the P1 receptor, the transduction mechanism associated with this response has not been clearly defined.

Cross talk between different classes of signaling molecules is an economic way for cells to expand their responses to a variety of chemical signals and neurotransmitters (for review, see Refs. 4, 40). For example, there is evidence of synergistic effects between ATP and adenosine to mobilize intracellular Ca2+ via the formation of d-myo-inositol (1,4,5)-trisphosphate [Ins(1,4,5)P3] in smooth muscle cell lines (14) and in Chinese hamster ovary cells transfected with the adenosine A1 receptor (24). However, very little is known about the presence of these types of interactions in ciliated cells.

In this work we studied the presence of P1 and P2 receptors and the transduction mechanisms associated with their stimulation of ciliary activity in hamster oviduct cultured ciliated cells. We characterized the sequence of molecular events induced by activation of both types of receptors. Furthermore, we show, for the first time, that both receptors are present in the same ciliated cell and that their transduction pathways are coupled to regulate ciliary activity in these cells.

MATERIALS AND METHODS

Reagents. ATP, β,γ-imido-ATP (AMP-PPN), β,γ-methylene-ATP (AMP-PCP), UTP, adenosine, 8-bromo-cAMP (8-BrcAMP); the poorly hydrolyzable and membrane-permeant cAMP analog, the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA), and Hanks' balanced salt solution were obtained from Sigma Chemical (St. Louis, MO); EDTA was obtained from Aldrich. The adenyl cyclase inhibitor 9-(tetrahydro-2-furyl) adenine (SQ-22536), the phospholipase C (PLC) inhibitor U-73122 and its inactive analog U-73343, the endoplasmic reticulum Ca2+-ATPase blocker thapsigargin, the Ins(1,4,5)P3 receptor inhibitor xestospongin C, and the PKC blocker GF-109203X were obtained from Calbiochem-Novabiochem International (San Diego, CA).

RESULTS

Spontaneous ciliary activity. Each ciliated cell of oviductal epithelium contains \( \sim 2 \times 10^5 \) cilia that acquire synchronized motion after 5 days in culture (46). The average spontaneous CBF measured was 11.55 ± 0.94 Hz (\( n = 60 \)). The frequency was highly dependent on temperature. A change of 5°C (from 35°C to 30°C) decreased the CBF from 11.35 ± 0.94 Hz to 6.25 ± 0.86 Hz (\( n = 5 \)). In our cultures the CBF was stable for at least 4 h, with a mean intercell coefficient of variation (20). This indicates that the CBF measured is a true basal activity and that it is not the result of a variable physiological state of the cells. For this reason we have used the term "spontaneous ciliary activity" for the activity measured in the absence of any exogenous stimulus.

Experimental procedure. All cultures used in the present study were observed with a Nikon Diaphot inverted microscope with a \( \times 40 \) objective lens. After 5 days in culture, cells showed spontaneous ciliary activity with a frequency range of 8–12 Hz. Ciliated cells were first equilibrated in Hanks' solution at 37°C for a period of 15 min, while ciliary activity was continuously monitored to determine average basal activity. Adenosine, ATP, ATP analogs, and the different chemical agents used were applied in Hanks' solution by rapid perfusion (<30 s) of the experimental chamber with a peristaltic pump. The CBF was measured up to 20 min after the addition of each analog. After each stimulation, cultures were washed three times to completely remove the added agents. No changes in CBF were observed during perfusion, ruling out any possible mechanical effect of perfusion on ciliary activity during the experiment. The CBF was recorded every 30 s, and experimental values were expressed as the percent change induced by the chemical agents on basal activity.

Data analysis. Statistical comparisons between different experimental conditions were made using analysis of variance of the arcsine-transformed data with the StatView program (StatView SE+Graphs). The criterion for a significant difference was a final value of \( F < 0.05 \). Data are expressed as means ± SE; \( n \) refers to the number of cultures analyzed.

The curves were fitted to logistic equations using the computer program Inplot (GraphPad Software for Science, San Diego, CA).

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CROSSED TALK BETWEEN ATP AND ADENOSINE RECEPTORS IN CILIATED CELLS

Fig. 1. A representative example of the time course for changes in ciliary beat frequency (CBF) induced by extracellular ATP. The ordinate values correspond to the difference, expressed as a percentage, between the CBF recorded during the experiment and the average basal activity obtained during the first 15 min. Ciliated cells stimulated with 100 µM ATP responded with an increased CBF. This response was composed of an initial transient component, lasting 2 min, followed by a sustained component, which decayed gradually. Similar responses were observed in all cells studied (n = 85). ATP was applied by perfusion for the period indicated by the bar. (defined as the percent change between the maximum and minimum frequency) of 2.33 ± 0.28 (n = 10).

ATP and related compounds increase the CBF. The spontaneous CBF increased after cell cultures were exposed to different chemical stimuli. Figure 1 shows a representative example of the response induced by external application of 100 µM ATP. After basal CBF was established, ciliated cells stimulated with different ATP concentrations evoked, within the first 30 s, a rapid increase in CBF. An initial transient component that reached a maximum of 83.45 ± 10.67% within 2 min was followed by a decay and then maintained a component that decayed gradually, with an average half-time of 9.5 ± 4.6 min (n = 85). We determined that desensitization of the receptor was minimal after a long exposure to ATP and that the CBF increase induced by ATP was fully recovered after 10 min of consecutive exposures to the agonist (data not shown).

To determine the class of P2 receptor involved in the ATP-induced response, we exposed cultures to different ATP analogs. As shown in Fig. 2, external ATP, AMP-PNP, AMP-PCP, and UTP increased the CBF in a dose-dependent manner. The time course of the frequency response to all ATP analogs was similar to that induced by ATP, with a transient and a maintained component (Fig. 2A). Dose-response curves showed that the increase in CBF was evident at 100 nM agonist concentrations and reached saturation at 100 µM, indicating that oviduct ciliated cells present a wide dynamic range of responses to these analogs, within approximately three logarithmic units (Fig. 2B). The stimulatory ranking efficacy of ciliary activity induced by ATP and related compounds was AMP-PNP > UTP > ATP > AMP-PCP. We also observed that the responses to AMP-PNP and ATP were biphasic, because concentrations of <100 µM produced a greater increase in CBF than that observed for higher concentrations (Fig. 2B). Dose-response curves between the concentrations from 1 nM to 100 µM were fitted using the equation $y = Max\left(1 + \left(ED_{50}/[A]\right)^{n_H}\right)$, where Max is the maximal effect, $ED_{50}$ represents the agonist concentration necessary to obtain the half-maximal effect, [A] is the agonist concentration, and $n_H$ is the Hill coefficient (data not shown). Analysis of the curves revealed an $ED_{50}$ of 10.2 and 12.9 µM for ATP and UTP, respectively. The $ED_{50}$ for AMP-PNP was 3.2 µM, about three times lower than those for ATP and UTP. The responses induced by ATP and its analogs thus imply the participation of P2Y$_2$ receptor. This is further supported by the observation that the ATP response was partially blocked by 100 µM suramin (data not shown) (16, 29).

Participation of PLC in the response to ATP and related compounds. To investigate whether PLC is an intermediary in the transduction cascade activated by ATP, we used U-73122, a known PLC inhibitor, or its inactive analog, U-73434 (5, 29). Pretreatment with U-73122 between 2 and 5 min before ATP addition reduced the ATP-induced increase from 80.20 ± 10.45% to 15.05 ± 5.23% of CBF in all cells examined (n = 6; Fig. 3A). In contrast, U-73434 was unable to prevent the ATP-induced response (n = 4; Fig. 3A). The experimental results are superimposed for greater clarity. To investigate whether the U-73122 inhibition of the ATP-induced response involves blocking the initial steps of the transduction cascade, three cultures of ciliated cells pretreated with U-73122 were incubated with 1 µM thapsigargin, an inhibitor of endoplasmic reticular calcium ATPase. As shown in Fig. 3B, thapsigargin increased CBF with an efficacy similar to that of ATP, suggesting that U-73122 produces its effect on the transduction cascade before calcium release from intracellular stores. We also examined the effect of U-73122 on the ciliary activity induced by ATP analogs. In all cases, U-73122 inhibited the CBF increase triggered by the agonists in a dose-dependent manner, although with differing sensitivities. Figure 3C shows the inhibition curves for ATP, UTP, and AMP-PNP. They show a sigmoidal relation that became evident at 10 nM U-73122. Analysis of the curves revealed similar inhibitory constants ($K_i$) for ATP and UTP, with values of 93 and 72 nM, respectively. For AMP-PNP, $K_i$ was 630 nM, about one order of magnitude higher than that for ATP or UTP. Figure 3C, inset, shows the effect of 1 µM U-73122 on the CBF increase induced by 100 µM AMP-PNP. We observed that higher concentrations of U-73122 are necessary to completely inhibit the agonist-induced CBF increase. The greater $K_i$ calculated for AMP-PNP might be related to the lower $ED_{50}$ and higher maximal effect on CBF induced by this compound compared with the other agonists (see Fig. 2B). Thus it is possible that AMP-PNP is more effective in activating PLC than ATP and UTP. Another possibility is that the AMP-PNP response is mediated by activation of more than one type of receptor that involves PLC activation. Altogether, these results suggest that PLC is an intermediary in the ATP-triggered transduc-
tion pathway and that its actions are probably mediated by increasing intracellular Ins(1,4,5)P_3 levels.

**Effect of Ca^{2+} on the ATP response.** Activation of P2 receptors has been associated with Ins(1,4,5)P_3-induced Ca^{2+} release from intracellular stores (3). To determine whether the ATP-dependent CBF increase involved a similar transduction system, we depleted the intracellular Ca^{2+} stores by treating cells with 1 mM thapsigargin for 20 min. In Fig. 4A, we show that thapsigargin induced a rise in CBF, which reached a maximum (90.25 ± 5.68%, n = 7) and then gradually diminished with an average decay half-time of 17.6 ± 3.2 min (n = 7). After ciliary activity returned to baseline, cells were stimulated with 100 μM ATP, which induced a small response. Similar results were obtained in six other cells, with an average increase of 13.00 ± 3.50%. The inability of extracellular ATP to induce a response after depleting intracellular Ca^{2+} stores and the similarity of the effects between ATP and thapsigargin strongly suggest that the ATP-induced increase in CBF involves a rise in intracellular Ca^{2+} concentration due to release of Ca^{2+} from internal stores. To determine the possible participation of an Ins(1,4,5)P_3 receptor as a mediator in the transduction pathway, we incubated ciliated cells with 5 mM xestospongin C, a potent blocker of the Ins(1,4,5)P_3 receptor (13). Figure 4B shows that there is a 70.00 ± 10.22% (n = 3) inhibition of the response to ATP in the presence of this blocker. This evidence suggests that the release of Ca^{2+} from internal stores is probably mediated by activation of Ins(1,4,5)P_3 receptors.

Reduction of external Ca^{2+} from 1 mM to 10 nM diminished the maximum effect induced by ATP from 85.00 ± 5.14% to 72.10 ± 0.70% (n = 3). The small reduction in the ATP response observed in low extracellular Ca^{2+} plus the large decrease in the
ATP response obtained in the presence of thapsigargin suggest that only a small component of the ATP-induced response is dependent on influx of extracellular Ca\(^{2+}\).

**Participation of PKC on the ATP response.** Having described the participation of PLC in the ATP-induced response, we proceeded to determine whether a PKC activation by the Ins(1,4,5)P\(_3\) cascade was involved in the ATP response. Ciliated cells were incubated with 2 \(\mu\)M GF-109203X, a blocker of PKC (41), for 8 min before ATP addition. Figure 5A shows that the ATP response is almost completely inhibited in the presence of the blocker \((n = 4)\). Furthermore, PMA, a PKC activator (30), transiently increases CBF on its own \((n = 4; \text{Fig. } 5\text{B})\). Moreover, coinubcation with ATP and PMA also increases CBF, with no effect on the transient response but with the maintained component showing a longer decay, with an average half-time of 16.7 ± 3.5 min \((n = 4; \text{Fig. } 5\text{B})\). To investigate the ability of ATP to evoke the same response with PKC downregulated by chronic treatment with PMA, we

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**Fig. 3.** Inhibitory effect of U-73122 on the CBF increase induced by ATP. A: a typical example of inhibition of the ATP-induced CBF increase with 1 \(\mu\)M U-73122 (●). This effect was reversed after removal of U-73122 from the experimental chamber. Similar results were obtained in 5 other cells. Exposure of ciliated cells to the inactive analog U-73343 did not inhibit the increase induced by ATP (○, \(n = 4\)). The time courses of these effects were superimposed for greater clarity. ATP, U-73122, and U-73343 were applied for the periods indicated by the bars. B: U-73122 inhibited the effect induced by ATP but was unable to inhibit the increase induced by thapsigargin (Thapsig; \(n = 5\)), indicating that U-73122 must act before Ca\(^{2+}\) is released from intracellular stores. C: inhibition of CBF increase induced by 100 \(\mu\)M ATP (●), UTP (○), and AMP-PNP (□) as a function of the U-73122 concentration. The plot represents the maximal inhibition, expressed as a percentage of CBF estimated at each U-73122 concentration. The experimental data (mean ± SE) derived from 5 different ciliated cells were fitted by means of the equation \(y = \text{Max}/[1 + (K_i/[I])^n_H]\), where Max is the maximal inhibition, \(K_i\) represents the inhibitor concentration necessary to obtain half-maximal inhibition, \([I]\) is the inhibitor concentration, and \(n_H\) is the Hill coefficient. Inset: the CBF inhibition induced by 1 \(\mu\)M AMP-PNP.

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**Fig. 4.** Time course of thapsigargin and xestospongin C on the CBF increase induced by ATP. A: depletion of intracellular Ca\(^{2+}\) stores with 1 \(\mu\)M thapsigargin partially inhibited the ATP-induced CBF increase. In the presence of inhibitor, an average increase of 13.00 ± 3.50% \((n = 7)\) was seen. B: response to 100 \(\mu\)M ATP (left) showed a 70.00 ± 10.20% inhibition in the presence of 5 \(\mu\)M xestospongin C (middle, \(n = 3\)). The response to ATP was recovered after removal of the inhibitor from the experimental chamber (right). Bars indicate the periods of cell stimulation.
incubated ciliated cells with 500 nM PMA for 16 h. We observed that the maximum increase in CBF induced by ATP was \( \frac{30}{n=2} \)%; 30% lower than the normal response \((n=2)\). These results suggest that PKC participates in the ATP response and that its effect might involve biochemical processes responsible for the maintained component of the CBF increase induced by ATP.

**Effect of adenosine on CBF.** The stimulatory effect of adenosine on oviductal ciliary activity is shown in Fig. 6. Adenosine increased CBF in a dose-dependent manner between 0.1 and 100 \( \mu M \), although the responses were smaller than those to ATP. It is important to highlight that only 35% of the treated cells responded to adenosine \((n=40)\). Low concentrations of adenosine (<10 \( \mu M \)) induced a slow increase in CBF that, after reaching a maximum, then decayed with a half-time constant of 2.65 ± 0.72 min \((n=5)\). Cells exposed to higher concentrations of adenosine (100 \( \mu M \)) showed a more sustained response to the agonist, with CBF remaining elevated for a period of time before returning to basal levels within an average half-time of 8.68 ± 1.17 min \((n=8)\). The responses of ciliated cells to a higher concentration of adenosine (1 mM) were smaller, with an increase of 23.42 ± 6.20% (data not shown). This suggests that the adenosine-induced response is also biphasic, like the ATP-induced response. This is shown in the dose-response curve in Fig. 6B.

To determine the class of P1 receptor, we exposed ciliated cells to CGS-21680, a known agonist of the A\(_{2a}\) subtype of the P1 receptor \((27)\). In all cells studied \((n=4)\), the agonist-induced response was similar to the adenosine-induced response, as shown in Fig. 6A. We demonstrated that ciliated cells of oviductal epithelium responded in a dose-dependent manner to adenosine.
In addition, we have observed that adenosine is unable to increase the intracellular Ca²⁺ concentration (2). These results suggest that the A₂a subtype of P1 receptor mediates the adenosine-induced response in ciliated cells.

Response to adenosine is mediated by adenylyl cyclase. In most tissues, P1 receptor subtypes (A₁, A₂a, A₂b, and A₃) mediate their effects via the modulation of adenylyl cyclase activity and, in part, by activation of PLC (A₁, A₂b, and A₃) (12, 37). To investigate whether the adenosine-induced response in oviductal ciliated cells involved the participation of adenylyl cyclase, cells that responded to adenosine were incubated with 1 mM SQ-22536, a known inhibitor of this enzyme (32), 20 min before a second adenosine application. As shown in Fig. 7A, the CBF basal activity did not change on application of SQ-22536, but this inhibitor did completely abolish the adenosine-induced response. A reduced response to adenosine was observed after the SQ-22536 was washed from the experimental chamber. Similar results were observed in five cells, suggesting that the effect induced by adenosine is mediated by activation of the adenylyl cyclase system. Following the usual strategy, we tested the effect of a cAMP analog on the CBF. Figure 7B shows the increase in CBF induced by 250 μM 8-Br-cAMP. The time course of the response was very similar to that observed with adenosine but with an activation slower than the response triggered by ATP. The dose dependence of this response is shown in Fig. 7B, inset. The effect induced by 8-Br-cAMP became significant at 1 μM and reached saturation point at 250 μM. The data presented suggest that cAMP participates as a second messenger in the adenosine-induced response.

Evidence for the participation of G protein in the adenosine response. No functional data are available that directly imply a role for G protein in the modulation of ciliary activity. However, it is likely that Gₛ protein couples P1 receptor (A₂) to adenylyl cyclase (37). To study this possibility, we incubated oviductal ciliated cells with cholera toxin (ChTX). It is known that 2 μg/ml ChTX for 6–8 h increases cAMP levels (42). We found that incubating for 4 h with 2 μg/ml ChTX potentiated the adenosine-induced response (Fig. 8). Preincubation with the toxin increased both the maximal response of 10 μM adenosine, from 48.00 ± 4.95% to 70.05 ± 10.20%, and the decay half-time, from 2.82 ± 0.63 to 16.45 ± 2.49 min ( n = 5). Moreover, we found that higher concentrations of ChTX (10 μg/ml) induced a response on their own. The toxin triggered a slow increase in CBF, followed by a slow decay lasting several minutes, which did eventually return to basal activity (Fig. 8, inset). The effect of ChTX is specific for adenosine, because pertussis toxin

![Fig. 7. Participation of the adenylyl cyclase system in the CBF increase induced by adenosine.](http://www.ajpcell.org) A: inhibitory effect of SQ-22536 on the CBF increase induced by adenosine. The time course is representative of the response induced by 100 μM adenosine (left). This response was completely inhibited when cells were bathed for 20 min in 1 mM SQ-22536 before adenosine was applied (middle). A new ATP response was evoked after the inhibitor was removed from the experimental chamber (right). Similar evidence was observed in 4 other experiments. B: time course of the response induced by 250 μM 8-bromo-cAMP (8-Br-cAMP; n = 10). Inset: plot of the CBF increase as a function of 8-Br-cAMP concentration. The values correspond to the average (mean ± SE) of 3–10 different cells. Bars indicate the bathing period of agonist incubation.

![Fig. 8. Effect of cholera toxin (ChTX) on the adenosine-induced increase in CBF.](http://www.ajpcell.org) Time course is representative of the potentiating effect of ChTX on the adenosine-dependent response. Left: time course of CBF increase induced by 10 μM adenosine (bar). This response was potentiated from 66% to 75% and did not return to basal activity (middle). Furthermore, higher concentrations of ChTX (10 μg/ml) induced a response on their own (inset).
failed to block this response \((n = 3, \text{ data not shown})\). These results are consistent with the suggestion that Gi protein-mediated coupling exists between P2 receptor activation and the change in the adenosine-induced CBF response.

Coupling between transduction mechanisms of P2 and P1 receptors. We have demonstrated that P2 and P1 receptors are present in oviductal ciliated cells and that both induce an increase in CBF through different pathways. To study a possible interaction between ATP and adenosine, we first investigated whether the same ciliated cell presents both P2 and P1 receptors. Figure 9 shows a cell that responded to both ATP and adenosine. Similar responses were also observed when the order of the agonists was reversed. Furthermore, in the same culture of ciliated cells, we also found cells that responded only to ATP, with no response to adenosine. These results support the idea that P2 and P1 receptors could be present in the same cell and make less likely the possibility that messengers secreted by other cells are responsible for the adenosine-induced increase of CBF. We then examined the interaction between both receptors by stimulating ciliated cells with ATP and adenosine simultaneously. Figure 10A shows a cell in which the CBF increased to 82% after being stimulated with 100 \(\mu\text{M}\) ATP. After being washed, the same cell exposed to 1 \(\mu\text{M}\) adenosine showed an increased CBF of only 20%. When both agonists were applied simultaneously, a CBF increase of up to 120% was observed, and the average decay half-time of the response increased considerably (Fig. 10A). The results obtained in five cells are shown in Fig. 10A, inset. Statistical analysis comparing the sum of the individual effects of ATP and adenosine (Sum) with the response induced by simultaneous application of both agonists, (ATP+Ad) showed that the response was more than additive \((P < 0.01, \text{ Fig. 10A, inset})\). A statistically significant synergistic interaction was observed in response to low doses of ATP and adenosine \((P < 0.05, \text{ Fig. 10B, inset})\). Concentrations of 100 nM ATP and 100 nM adenosine, which generally were unable to increase CBF, caused a clear increase in CBF when combined (Fig. 10B, inset; compare Sum and ATP+Ad). The same effect was also observed with ATP and 8-BrcAMP (Fig. 10C), suggesting that this synergism involves the transduction pathways of the responses to agonists. To evaluate this hypothesis, we studied the effect of SQ-22536 (adenylcyclase inhibitor) on the ATP-induced response and the effect of U-73122 (PLC inhibitor) on the adenosine-induced response. Figure 10, D and E, shows representative examples of the results obtained in both conditions. Figure 10D shows one cell, which under normal conditions responds to ATP. In the presence of 1 mM SQ-22536, the ATP-induced response was reduced by 60% (58 ± 10.52; \(n = 6\)). The subsequent application of 1 \(\mu\text{M}\) thapsigargin only increased CBF by 30%, less than the effect obtained in the absence of SQ-22536 (compare Fig. 4A and Fig. 10D), suggesting that intracellular cAMP basal levels are important in generating the ATP-response. The ATP response was partially recovered after the ciliated cells were washed with Hanks’ solution that was free of SQ-22536 \((n = 6)\). Similar results were obtained with nonhydrolyzable analogs of ATP \((n = 2)\). Figure 10E shows that 1 \(\mu\text{M}\) U-73122 inhibited the adenosine-response by 40% \((36.25 ± 8.30; n = 5)\). In contrast to the previous experiment, U-73122 was unable to abolish the response to thapsigargin (compare Fig. 10, D and E) and 8-BrcAMP (data not shown).

Our results demonstrate that ATP and adenosine have a synergistic effect on the control of ciliary that might occur through the activation of cross talk mechanisms in ciliated cells.

DISCUSSION

Although the effects of extracellular ATP and adenosine on ciliary activity are well established (10, 15, 26, 33, 40, 45, 49), few studies have been aimed at understanding exactly how the signals produced by receptor activation are integrated at the cellular level to control ciliary activity. In the present work, we demonstrate the presence of P2 and P1 receptors in oviductal ciliated cells and show how ciliary activity may be regulated through interactions between their respective signal transduction pathways.

Oviductal ciliated cells responded with a dose-dependent CBF increase to the application of external ATP. Similar responses were obtained with AMP-PNP and AMP-PCP (nonhydrolyzable analogs of ATP), suggesting that neither the ATP-sensitive increase in CBF nor the decay of the response is dependent on ATP hydrolysis. Processes such as receptor desensitization or adaptation could be responsible for the decay of the ATP-induced effect.

The evidence provided here is consistent with the idea that ATP acts by binding to membrane receptors. The stimulatory ranking efficacy of ATP and analogs (AMP-PNP > UTP ≥ ATP > AMP-PCP) plus the observation that UTP induced an effect similar to that of ATP suggest that the ATP-induced response is trig-
Fig. 10. Cross talk between P2 and P1 receptors. Graphs show the synergistic effect between ATP and adenosine and the cross talk between their signal transduction pathways. A: increase in CBF elicited by ATP (left), adenosine (middle), and simultaneous application of both agonists (right). Data (means ± SE) from 5 experiments are shown in the inset. A statistical analysis comparing the algebraic sum of the individual effects for 100 μM ATP and 1 μM adenosine (Sum) with the observed response when both agonists were added simultaneously (ATP+Ad) showed that the effect was synergistic and not additive (ANOVA test, *P < 0.01). B: conditions similar to those shown in A but at concentrations of ATP (100 nM) and adenosine (100 nM) that were usually unable to modify the CBF. Data (means ± SE) from 5 experiments are shown in the inset. A comparison of the Sum and ATP+Ad demonstrates that the synergistic interaction is highly significant (ANOVA test, **P < 0.005). C: synergism between ATP and 8-BrcAMP (cAMPα). Recordings obtained from 100 μM ATP and 30 μM 8-BrcAMP and from the combination of these concentrations are shown. A statistical analysis of the data is presented in the inset. A comparison of the Sum and ATP+cAMPα shows that the effect was also more than additive (ANOVA test, **P < 0.005). Data (means ± SE) are from 3 experiments. D: effect of SQ-22536, an inhibitor of the cAMP pathway, on the ATP response. SQ-22536 produced a 60% inhibition of the ATP response and a 30% inhibition of the thapsigargin response. Similar results were observed in 5 different cells. E: effect of U-73122, an inhibitor of the inositol (1,4,5)-trisphosphate pathway, on the adenosine response. The U-73122 produced a 40% inhibition of the adenosine response but was unable to abolish the thapsigargin effect. Similar results were obtained in 4 other cells. Bars indicate the period of agonist stimulation.
gered via P2Y2 receptors. Gheber et al. (15) have pharmacologically detected the presence of P2 receptors in ciliated cells from frog esophagus epithelium. On the basis of evidence that ADP and ATP are similarly potent in their ability to stimulate CBF, they suggest that P2Y4 receptors, according to the new classification, are present in ciliated cells from the airway epithelium. In 10 oviductal cells that responded to ATP and UTP, we never found an effect of ADP (data not shown), confirming our suggestion that the increase in CBF is via a P2Y2 receptor (16). The observation that suramin did not completely block the ATP response suggests the possibility that P2 receptors in oviductal ciliated could be either P2Y2 and P2Y4 receptors. Further experiments using RT-PCR are needed to answer this question.

The molecular events associated with ATP stimulation in ciliated cells have been poorly studied, and most of our knowledge about these effects comes from other cell types (for review, see Ref. 8). In this study we found that U-73122, a known PLC inhibitor, reversibly abolished the effect induced by ATP and its analogs, suggesting that PLC is part of the transduction cascade triggered by ATP. However, thapsigargin, an inhibitor of the Ca2+-ATPase that depletes the Ca2+ intracellular stores, also inhibited the response to this nucleotide, indicating that Ca2+ released from internal stores probably participates in the CBF increase triggered by ATP. This confirms our previous finding that Ca2+ is involved as an intermediary in the ATP-activated pathway (45). Furthermore, Ca2+ is probably released via Ins(1,4,5)P3 receptors, because xestospongion C, an Ins(1,4,5)P3 receptor blocker, inhibited the ATP effect by 70%. The CBF inhibition induced by thapsigargin and xestospongion C was not complete, indicating that a component of this response is due to an influx of extracellular Ca2+. Ciliated cells exposed to low extracellular Ca2+ concentrations (10 nM) showed a decreased response to ATP (13%) compared with controls (1 mM Ca2+). Korngreen and Priel (18), in a study of measurement of intracellular Ca2+ and CBF in rabbit ciliated airway epithelium, have demonstrated that extracellular Ca2+ participates in the ATP-dependent response.

In many cell types there is evidence that PLC and PKC are part of the transduction mechanism activation for a variety of stimuli (25, 35). Our results show that PKC activation by PMA induced a CBF increase. A similar increase in CBF has been observed in frog esophagus epithelium, after exposure to 12-O-tetradecanoylphorbol 13-acetate, a PKC activator (20). Moreover, coinoculation of cells with ATP and PMA potentiates the maintained component of the response, giving a slower decay compared with the control. The response was also diminished by a selective PKC inhibitor (GF-109203X) and by enzyme downregulation. These findings suggest that PKC modulates biochemical processes that might be responsible for the duration of ciliary stimulation produced by ATP.

Exposure to adenosine also increased the CBF in oviductal ciliated cells, indicating the possible presence of the A2a subtype of P1 receptors, because CGS-21680, an agonist of subtype A2a receptor, induced a response similar to that of adenosine. Similar results have been obtained with adenosine in other preparations (49). Tamaoki et al. (39) found that high concentrations of adenosine (1 mM) diminished basal ciliary activity in cultured rabbit tracheal epithelium, and they suggested that this effect was mediated by adenosine receptors. In oviduct, although the adenosine response was biphasic and high concentrations (1 mM) of agonist induced a response that was smaller than that to micromolar concentrations, we never observed an inhibition of the basal CBF in cells exposed to 1 mM adenosine. This difference may be due to the ciliated cell type used in both studies. Ca2+ mobilization has been used to distinguish between A2a and A2b receptor subtypes. An increase in intracellular Ca2+ concentration occurs after activation of A2b adenosine receptors in canine BR mastocytoma (1) and HEK-293 cells transfected with recombinant human subtype A2b of the adenosine receptor (21). Recent observations in our laboratory indicate that adenosine does not increase intracellular free Ca2+ concentration in oviductal ciliated cells (2). This observation, plus the fact that CGS-21680, an agonist of subtype A2a of P1 receptors, produced a response similar to that induced by adenosine, supports the idea that A2a receptors are present in oviductal ciliated cells.

The intracellular molecular events activated by binding of adenosine to its membrane receptor in ciliated cells are poorly understood. Our finding that micromolar and millimolar concentrations of SQ-22536 completely abolished the ciliated cell response to adenosine suggests the participation of an adenylyl cyclase in the transduction pathway of P1 receptor. In addition, ciliated cells treated with 8-BrcAMP showed a dose-dependent increase in CBF. The dose-response curve was similar to that previously reported by Tamaoki et al. (38) in rabbit tracheal epithelial cells. These results suggest that cAMP could be the second messenger involved in the adenosine response. We have also demonstrated here, for the first time, that Gs protein couples the P1 receptor with adenylyl cyclase in ciliated cells. In the presence of 2 μg/ml ChTX, cells showed a greater adenosine-induced increase in CBF than in the absence of this toxin. Moreover, at high concentrations ChTX induced a response on its own. From these results, we propose a mechanism by which stimulation of P1 receptor by adenosine triggers a Gs protein-mediated biochemical cascade that increases intracellular levels of cAMP and that could enhance the CBF through a mechanism as yet unknown. On the basis of evidence obtained in Paramecium cilia, it has been proposed that cAMP-dependent phosphorylation of ciliary dyneins plays a role in the regulation of ciliary motility (48). A similar mechanism may be present in oviductal ciliated cells, although further studies are necessary to identify the ciliary proteins that may be phosphorylated or dephosphorylated to better understand the mechanism involved in control of oviduct ciliary activity.
Mechanisms of cross talk in ciliated cells are inadequately understood. However, our tissue culture system of oviductal ciliated cells proved to be a good model for investigation of this phenomenon. First of all, we suggest that ciliated cells have both P2 and P1 receptors, because cells responded to both ATP and adenosine (Fig. 9). Moreover, simultaneous application of both agonists produced a higher increase in the CBF than the sum of the individual responses. These results provide the first evidence that the same ciliated cell presents P2Y<sub>2</sub> and the A<sub>2a</sub> subtype of P1 receptors and that both receptors seem to act synergistically to increase CBF. A synergistic interaction between ATP receptor and adenosine A<sub>1</sub> receptors in Ins(1,4,5)P<sub>3</sub> formation and the mobilization of intracellular Ca<sup>2+</sup> has been seen in smooth muscle cells (14) and in Chinese hamster ovary cells transfected with the human adenosine A<sub>1</sub> receptor (24). On the basis of the observation that a synergism exists between ATP and 8-BrcAMP, plus the facts that the ATP response was inhibited by SQ-22536 and the adenosine response was abolished by U-73122, we propose that the synergic action observed in these cells is produced by a cross talk mechanism that could operate at the signal transduction pathway level. Even though there are recent reports of cross talk in different cell types (4, 17, 22, 24, 34), we are the first to report evidence of this mechanism in ciliated cells. This cross talk mechanism could be of greater importance in cellular physiology, because lower agonist concentrations would be able to induce a cellular response. Although we did not determine the target molecules involved in this cross talk mechanism, there is evidence that Ca<sup>2+</sup> and protein kinases might be good candidates. For example, it is known that intracellular Ca<sup>2+</sup> stimulates adenyl cyclase through a variety of mechanisms, including interactions with protein kinases (6). We also know that ATP increases intracellular Ca<sup>2+</sup> concentration in oviductal ciliated cells (45), and thus it is possible that the synergism in these cells requires basal cAMP levels accompanied by Ca<sup>2+</sup>-dependent kinase activation. Post et al. (31) have demonstrated that P2Y receptor agonists enhance cAMP production in Madin-Darby canine kidney epithelial cells. Although we have no evidence that ATP increases cAMP production in ciliated cells, we observed a synergistic and sustained CBF increase in cells simultaneously stimulated with low doses of ATP and 8-BrcAMP (Fig. 10C). Moreover, the reduction of the ATP response in the presence of SQ-22536 supports the idea that basal cAMP levels are important for the CBF increase induced by ATP. In a similar way, the reduction in the response to adenosine by a PLC blocker could modify basal levels of other mediators involved in the transduction pathway, such as PKC, which has been shown to affect adenosine receptor subtype A<sub>2a</sub> (28). Another possible clue to the underlying mechanisms of cross talk present in ciliated cells could come from that described in Chinese hamster ovary cells transfected with V<sub>1a</sub> and V<sub>2</sub> vasopressin receptors (17). In that study the authors suggested that the V<sub>2</sub>-induced cAMP accumulation is potentiated by the stimulation of the V<sub>1a</sub> receptor transduction pathway, a process that occurs essentially via PKC, which produces an increase in G<sub>q</sub> coupling with adenylyl cyclase (17). More experiments are clearly required to characterize the molecules involved in the cross talk mechanism present in ciliated cells.

In summary, the major finding of the present study is the clear functional demonstration of the presence of both P2Y<sub>2</sub> and the A<sub>2a</sub> subtype of P1 receptors in the same ciliated cell. We also characterize the signal transduction systems associated with activation of these two receptor types and propose that a mechanism of cross talk could be controlling ciliary activity at different levels of the signal transduction pathways.

This work was supported by Fondo Nacional de Ciencia y Tecnología 1971260 and 8980008.

REFERENCES


CROSS TALK BETWEEN ATP AND ADENOSINE RECEPTORS IN CILIATED CELLS


