Plasma and intracellular membrane inositol 1,4,5-trisphosphate receptors mediate the Ca\(^{2+}\) increase associated with the ATP-induced increase in ciliary beat frequency

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Barrera, Nelson P., Bernardo Morales, and Manuel Villalón. Plasma and intracellular membrane inositol 1,4,5-trisphosphate receptors mediate the Ca\(^{2+}\) increase associated with the ATP-induced increase in ciliary beat frequency. Am J Physiol Cell Physiol 287: C1114–C1124, 2004. First published June 2, 2004; 10.1152/ajpcell.00343.2003.—An increase in intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) has been shown to be involved in the increase in ciliary beat frequency (CBF) in response to ATP; however, the signaling pathways associated with inositol 1,4,5-trisphosphate (IP3) receptor-dependent Ca\(^{2+}\) mobilization remain unresolved. Using radioimmunoassay techniques, we have demonstrated the appearance of two IP3 peaks occurring 10 and 60 s after ATP addition, which was strongly correlated with a release of intracellular Ca\(^{2+}\) from internal stores and an influx of extracellular Ca\(^{2+}\), respectively. In addition, ATP-dependent Ca\(^{2+}\) mobilization required protein kinase C (PKC) and Ca\(^{2+}\)/calmodulin-dependent protein kinase II activation. We found an increase in PKC activity in response to ATP, with a peak at 60 s after ATP addition. Xestospongin C, an IP3 receptor blocker, significantly diminished both the ATP-induced increase in CBF and the initial transient [Ca\(^{2+}\)]\(_i\) component. ATP addition in the presence of xestospongin C or thapsigargin revealed that the Ca\(^{2+}\) influx is also dependent on IP3 receptor activation. Immunofluorescence and confocal microscopic studies showed the presence of IP3 receptor types 1 and 3 in cultured ciliated cells. Immunogold electron microscopy localized IP3 receptor type 3 to the nucleus, the endoplasmic reticulum, and, interestingly, the plasma membrane. In contrast, IP3 receptor type 1 was found exclusively in the nucleus and the endoplasmic reticulum. Our study demonstrates for the first time the presence of IP3 receptor types 1 and 3 in the plasma membrane in ciliated cells and leads us to postulate that the IP3 receptors can directly trigger Ca\(^{2+}\) influx in response to ATP.

transduction mechanisms; P2Y receptor; calcium influx

CILIATED CELLS FROM OVUDUCTAL MUCOSA play an important role in the control of the mucociliary transport velocity of gametes and embryos (27). These cells modify their ciliary beat frequency (CBF) in response to a variety of chemical, electrical, and mechanical signals. For example, extracellular ATP is a powerful activator of CBF in cells derived from rabbit oviduct (46), rabbit trachea (19, 42), human trachea (21), and frog palate and esophagus (12, 39, 50), and an inhibitor of CBF in rat brain ependymal cells (31). Recently, we demonstrated that the increase in CBF induced by ATP in ciliated oviductal cells was mediated by P2Y\(_2\) receptor activation (28). Furthermore, we provided evidence that stimulation of ciliary activity by ATP requires phospholipase C (PLC) activation, Ca\(^{2+}\) mobilization by Ca\(^{2+}\) released from intracellular stores through inositol 1,4,5-trisphosphate (IP3) receptor activation and Ca\(^{2+}\) influx, and protein kinase C (PKC) activation. It is well known that in ciliated cells, a rise in intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) through release from intracellular stores results in an increase in CBF (15, 19, 28, 42, 46). Furthermore, airway epithelial cells demonstrate Ca\(^{2+}\) wave propagation that is mediated by the activation of IP3 receptors (14, 37). However, the role and subcellular distribution of IP3 receptors involved in the ATP-dependent [Ca\(^{2+}\)]\(_i\) increase in ciliated cells remain unknown.

In mammals, at least three IP3 receptor types (types 1, 2, and 3) are encoded by three distinct genes (2, 38), and the expression of the IP3 receptors has been reported in ciliated cells from both rat olfactory cilia (8) and murine oviduct (29). IP3 receptors participate in Ca\(^{2+}\) signaling by mediating intracellular Ca\(^{2+}\) release (16) and regenerative Ca\(^{2+}\) signals (9). Interestingly, the type 3 receptor mediates regulation of Ca\(^{2+}\) flux across the plasma (34, 35, 44) and nuclear (40) membranes.

IP3 receptors can be regulated in a number of ways. They contain binding sites for Ca\(^{2+}\), and elevation in free [Ca\(^{2+}\)] from nanomolar to micromolar concentrations modifies receptor properties (36). Differential Ca\(^{2+}\) sensitivity of Ca\(^{2+}\) activation sites between IP3 receptor types 1 and 3 (5, 25) and a hypothetical differential subcellular localization and expression of IP3 receptor types in the same ciliated cell could evoke complex Ca\(^{2+}\) responses to extracellular agonist stimulation. In addition, several cytosolic factors, including Ca\(^{2+}\)/calmodulin (CaM) (1), and phosphorylation by protein kinase A (PKA) (51), PKC (10), and CaM-dependent protein kinase II (CaMKII) (53) can modulate the activity of IP3 receptors. However, whether these factors are associated with the ATP transduction pathways in oviductal ciliated cells is an unresolved issue.

Although the activating effect of ATP on CBF has been demonstrated in ciliated cells, the presence and mechanisms of activation of IP3 receptor associated with the ATP-dependent control of CBF are still not completely understood. In the present study, we show that the participation of the extracellular and intracellular Ca\(^{2+}\) reservoirs in the ATP response is dependent on IP3 receptor activation and that PKC and CaMKII are involved in Ca\(^{2+}\) mobilization. Furthermore, we have analyzed the quantitative and temporal relationships be-
tween ATP-induced CBF increase and changes in [IP3] and [Ca2+]. In addition, we have demonstrated the differential subcellular localization of IP3 receptor types 1 and 3 in the same oviductal ciliated cell, showing that only the type 3 receptor is present in plasma membrane. Finally, we discuss the relationship between timing and subcellular localization of IP3 receptor activity in ciliated cells and suggest that both receptor types can play specific roles in the induction of the ATP-dependent [Ca2+] increase.

METHODS

Chemicals and solutions. ATP, the PKC activator phorbol 12-myristate 13-acetate (PMA), EGTA, and Hanks’ balanced salt solution were obtained from Sigma-Aldrich (St. Louis, MO). Fura 2-AM was obtained from Molecular Probes (Eugene, OR). The PLC inhibitor U-73122, the IP3 receptor inhibitor xestospongin C, the PKC blocker bisindolylmaleimide I (GF-19023X), the CaM blocker W-7, the CaMKII inhibitor peptide 281–309 (CBP), and the Ca2+ pump ATPase blocker thapsigargin were obtained from Calbiochem-Novabiochem International (San Diego, CA). Stock solutions of ATP, W-7, and CBP were prepared in water (pH 7.4) and diluted in Hanks’ buffer just before use. In addition, the CBP solution was added to the Rose chamber in a hypotonic medium (~35% reduction of normal osmolality, ~290 mosM, in Hanks’ buffer) during the period of incubation. After this incubation, this solution was changed to Hanks’ buffer. All other reagents were dissolved in dry dimethyl sulfoxide (DMSO) at a final concentration of 0.1%. At this concentration, DMSO had no effect on CBF.

Culturing of ciliated cells from hamster oviductal epithelium. Experiments were performed in primary cultures of hamster oviductal epithelium obtained using the procedure previously described by Verdugo et al. (45). Adult (2–3 mo old) hamsters were anesthetized with 30% halothane and then decapitated. The ovriducts were removed and placed in Hanks’ solution (Sigma-Aldrich) at pH 7.4. The distal portion of the oviduct (i.e., the fimbria) was cut into small (4–8 mm2) pieces and placed in N-hydroxysuccinimide culture medium (in mM: 137 NaCl, 5.09 KCl, 1.14 NaH2PO4, 0.18 KH2PO4, 0.923 MgCl2, 0.91 CaCl2, 4.07 NaHCO3, 21.5 glucose, and 0.2 glutamine). The tissue was homogenized using the XL Microson ultrasonic cell disruptor (Heat Systems Ultrasonics, Farmingdale, NY). Briefly, each homogenate was centrifuged for 10 min at 2,000 g. The supernatant was collected and the TCA was extracted using 2 volumes of a 3:1 solution of 1,1,2-trichloro-1,2,2-trifluoro-ethane-trioctylamine. The resulting aqueous phase, which contains IP3, was removed using a transfer pipette. Samples were maintained at ~70°C until analysis. The IP3 concentration ([IP3]) in 20 μg of protein from oviductal ciliated cells was determined using a commercial radioiodide assay kit (NEN Life Sciences, Boston, MA). The [IP3] was expressed as picomoles of IP3 per microgram of protein. Epithelial protein content was determined using the Bradford method (3). A recovery of 98% was observed after the addition of a known amount (1 pmol) of IP3 to the culture medium. The sensitivity of the assay was 0.05 pmol IP3. In all cases, cross reactivity with inositol 1,4-bisphosphate, inositol 1,3,4-trisphosphate, and inositol 1,3,4,5-tetrakisphosphate was <2%.

Extraction and quantification of PKC activity. Oviductal ciliated cells obtained by mechanical extraction of the epithelium of fimbria were placed in Hanks’ medium at 37°C in an atmosphere of 5% CO2 and incubated with ATP. The reaction was stopped with 1 M cold trichloroacetic acid (TCA). The TCA precipitate was collected by centrifugation at 2,000 g for 10 min at 4°C for PKC extraction. The supernatant was collected and the TCA was extracted using 2 volumes of a 3:1 solution of 1,1,2-trichloro-1,2,2-trifluoro-ethane-trioctylamine. The resulting aqueous phase, which contains IP3, was removed using a transfer pipette. Samples were maintained at ~70°C until analysis. The IP3 concentration ([IP3]) in 20 μg of protein from oviductal ciliated cells was determined using a commercial radioiodide assay kit (NEN Life Sciences, Boston, MA). The [IP3] was expressed as picomoles of IP3 per microgram of protein. Epithelial protein content was determined using the Bradford method (3). A recovery of 98% was observed after the addition of a known amount (1 pmol) of IP3 to the culture medium. The sensitivity of the assay was 0.05 pmol IP3. In all cases, cross reactivity with inositol 1,4-bisphosphate, inositol 1,3,4-trisphosphate, and inositol 1,3,4,5-tetrakisphosphate was <2%.

Immunocytochemical localization of IP3 receptors in cultured ciliated cells. Immunofluorescence and confocal microscopy were used to determine the cellular localization of the IP3 receptors. Cultured ciliated cells were fixed for 30 min at room temperature in 3% paraformaldehyde in PBS containing 2 mM MgCl2 and 0.2 mM CaCl2 (PBSCaMg). Samples were washed three times in PBS/CaMg. Next, ciliated cells were permeabilized with 0.2% Triton X-100 in PBSCaMg for 10 min. Samples were washed four times in PBS containing 0.2% gelatin (PBS-gel) and incubated overnight at 4°C with the purified primary antibodies: anti-IP3 receptor type 1 (polyclonal; Affinity BioReagents, Golden, CO) and/or anti-IP3 receptor type 3 (monoclonal; BD Transduction Laboratories, San Diego, CA), dilution 1:50. Samples were washed four times in PBS-gel and then incubated at 37°C for 10 min with either secondary antibody Cy3-conjugated, affinity-purified goat anti-rabbit IgG (Jackson Immuno-Research Laboratories, West Grove, PA), dilution 1:50, or with Cy3-conjugated, affinity-purified goat anti-mouse IgG (Jackson Im-
RESULTS

\[ \text{[Ca}^{2+}]_{i} \] increase induced by extracellular ATP in ciliated cells. We previously suggested that the increase in CBF induced by ATP in oviductal ciliated cells involves \text{Ca}^{2+} mobilization from intracellular reservoirs and extracellular medium (28). We therefore first focused on quantifying and characterizing the effect of ATP on \([\text{Ca}^{2+}]_{i}\), in single ciliated cells from the oviduct. Figure 1A shows the time course of the effect of extracellular ATP on \([\text{Ca}^{2+}]_{i}\). After the addition of 100 \(\mu\)M ATP, we observed two components of the response. First, the transient \([\text{Ca}^{2+}]_{i}\), increase reached the maximum average value of 387.8 \pm 32.0 \(\text{nM}\) over basal \([\text{Ca}^{2+}]_{i}\), (70.6 \pm 5.4 \(\text{nM}\); data not shown) within 10.8 \pm 2 s, and then the response diminished to a plateau of 56.2 \pm 7.0 \(\text{nM}\), which was higher than basal levels. To analyze the effect of ATP on \([\text{Ca}^{2+}]_{i}\), we fitted the response decay to a simple exponential equation with the form \(\text{[Ca}^{2+}]_{i} = a + b e^{-\text{t/H}}\), where \(a\) and \(b\) represents the peak value, \(a\) is the plateau value, and \(H\) is the decay time constant. Figure 1B shows a distribution of the peak and plateau values (describing the \([\text{Ca}^{2+}]_{i}\), increase) in relation to ATP concentration. Concentration-response curves of peak and plateau values between 1 \(\text{mM}\) and 100 \(\mu\)M ATP were fitted using the equation \(y = \text{max} \{1 + (\text{EC}_{50} [\text{A}]^{-1}) n_{H} \}^{-1}\), where max is the maximal effect, \(\text{EC}_{50}\) represents the agonist concentration necessary to obtain the half-maximal effect, \([\text{A}]\) is the ATP concentration, and \(n_{H}\) is the Hill coefficient. Analysis of the curves revealed \(\text{EC}_{50}\) values of 6.45 \pm 0.68 \(\text{\muM}\) for the peak and 83.1 \pm 1.02 \(\text{\muM}\) for the plateau. Student’s \(t\)-test analysis demonstrated statistically significant differences between the two values \((P < 0.05)\), suggesting a higher efficacy of ATP in the generation of the transient component. Taken together, these results demonstrate a concentration-dependent \([\text{Ca}^{2+}]_{i}\), response for the two components.

\(\text{IP}_3\) receptor activation during \([\text{Ca}^{2+}]_{i}\), increase induced by ATP. Recently, we found that a reduction in the extracellular \(\text{Ca}^{2+}\) concentration \(([\text{Ca}^{2+}]_{o}\)) from 1 \(\text{mM}\) to 10 \(\text{nM}\), as well as inhibition of \(\text{IP}_3\) receptor activation, reduced the CBF increase induced by ATP (28). We therefore analyzed \(\text{IP}_3\) receptor activity during the ATP-induced \([\text{Ca}^{2+}]_{i}\), increase. Figure 2A shows that a reduction of \([\text{Ca}^{2+}]_{i}\), to 10 \(\text{nM}\) (using EGTA)
resulted in a 94.4 ± 1.7% inhibition of the plateau value of [Ca$^{2+}$], ($P < 0.05$), without causing significant changes in the peak value. In addition, the CBF and basal values of [Ca$^{2+}$] were not modified in the Ca$^{2+}$-free medium. The maximum value of the CBF increase induced by ATP was diminished by only 15.3 ± 2.4% in the Ca$^{2+}$-free medium (28); however, there was a significant reduction in the peak value, suggesting that the Ca$^{2+}$ influx is not required to trigger the ATP-dependent ciliary response but may be needed to maintain or amplify it. On the other hand, as shown in Fig. 2B, the first component of the [Ca$^{2+}$] increase induced by ATP was significantly diminished in the presence of xestospongin C, an inhibitor of IP$_3$ receptor activation (11). In the presence of 5 μM xestospongin C added 10 min before ATP application, the peak value was diminished by 81.9 ± 4.5% ($P < 0.05$), and there was also a reduction in the plateau value of 58.8 ± 3.6% ($P < 0.05$) compared with the control response (note also that 35% of the treated cells exhibited total inhibition). The use of Ca$^{2+}$-free medium did not change the $\tau$ value ($\tau = 25.3 \pm 1.7$ s) compared with control ($\tau = 23.1 \pm 1.3$ s), suggesting that the origin of the first component of the response and its decay is accounted for by Ca$^{2+}$ mobilization from intracellular Ca$^{2+}$ stores induced by IP$_3$ receptor activation. Because thapsigargin-dependent intracellular Ca$^{2+}$ stores participate in the ATP-induced CBF response (28), we used this Ca$^{2+}$ ATPase pump blocker to study the effect of IP$_3$ receptor activation on the [Ca$^{2+}$] increase. When the ciliated cells were preincubated with 1 μM thapsigargin, the addition of 100 μM ATP caused a smaller increase in [Ca$^{2+}$], with respect to the control (Fig. 2C). However, the effect of 100 μM ATP on [Ca$^{2+}$], was almost completely abolished after thapsigargin addition in the presence of extracellular EGTA (10 nM extracellular Ca$^{2+}$; Fig. 2C). Interestingly, abolition of the ATP effect ($P = 0.001$) was also observed when ciliated cells were preincubated with thapsigargin followed by treatment with 5 μM xestospongin C for 10 min before ATP application (Fig. 2D). These results...
suggest an IP₃ receptor activation-dependent Ca²⁺ influx. Taken together, these results indicate that the first component of the [Ca²⁺]ᵢ increase depends on IP₃ receptor activation and that this component is required to initiate the ATP-induced increase in CBF. Moreover, it is possible that the Ca²⁺ influx is mediated directly by IP₃ receptor activation. On the other hand, Morales et al. (28) obtained a complete inhibition of the CBF increase induced by ATP in the presence of a PLC inhibitor, U-73122 (6, 32). Because the PLC activation-dependent [IP₃]ᵢ increase should involve the participation of an IP₃ receptor, we determined whether PLC inhibition blocked the [Ca²⁺]ᵢ increase. Figure 2E demonstrates complete inhibition of ATP-induced [Ca²⁺]ᵢ increase in the presence of 1 µM U-73122, suggesting that both components of the response require PLC activation. Interestingly, the delay in the response evoked by ATP in the presence of the different pharmacological agents used is similar to that in control ATP responses, suggesting the participation of the same ATP-activated transduction pathways in the different experimental approaches. Taken together, these results suggest that an initial component, which is dependent on IP₃ receptor activation, initiates the CBF increase. Subsequently, Ca²⁺ influx probably induced by IP₃ receptor activation is involved in the maintenance or amplification of the CBF response induced by ATP.

Increased IP₃ synthesis in response to ATP and the participation of PKC in the [Ca²⁺]ᵢ increase. Because the PLC pathway and IP₃ receptor activation are responsible for the [Ca²⁺]ᵢ increase induced by ATP, we studied the effect of ATP on IP₃ synthesis in oviductal ciliated cells. Figure 3A shows the time course of the [IP₃]ᵢ response induced by 100 µM ATP (n = 12). We observed a maximum increase in [IP₃]ᵢ at 10 s (66.95 pmol IP₃·µg⁻¹ protein) after ATP addition, followed by a mean response between 30 s and 4 min, which was 9.5 ± 1.5-fold lower than the maximum response (P < 0.05). It is important to emphasize that a significant [IP₃]ᵢ increase had already started 6 s after ATP addition. Consistent with the participation of PLC in the ATP response, a complete block in the [IP₃]ᵢ increase was observed when the ciliated cells were preincubated with 1 µM U-73122 (n = 5) in the presence of ATP. Figure 3B demonstrates a concentration-response curve for [IP₃]ᵢ in the presence of ATP. The same equation used in Fig. 1C fitted the data between 1 nM and 100 µM. EC₅₀ was 0.29 ± 0.5 µM of the [IP₃]ᵢ increase, which is ~20-fold lower than the EC₅₀ of the peak value of the [Ca²⁺]ᵢ increase. In addition, the EC₅₀ for CBF increase induced by ATP was 10.2 µM (28). These results suggest that the sensitivity of the ATP transduction pathways tends to decrease along with an advance in the signaling cascade. However, knowledge of the generation of second messengers at each time point during the response would be required to determine how dynamic responses involved in the ATP transduction pathways are sequentially activated. In support of the idea of a strong relationship between IP₃ synthesis and Ca²⁺ mobilization, the maximum change in IP₃ synthesis had a high correlation (r = 0.9501) with the peak Ca²⁺ value (Fig. 3C). We also observed that the second, lower [IP₃]ᵢ increase at 60 s had a high correlation (r = 0.9615) with the plateau value (Fig. 3C), suggesting that both the intracellular Ca²⁺ mobilization from internal stores and the Ca²⁺ influx can be related to two IP₃ peaks occurring at different times. To summarize the effect of ATP on ciliary activity, in Fig. 3D, we superimposed the time courses of CBF, [Ca²⁺]ᵢ, and [IP₃]ᵢ increases induced by 100 µM ATP. Interestingly, these three responses had similar time courses (P < 0.05; ANOVA), and, as previously postulated, we observed an order in the sequence of responses to ATP, beginning with an [IP₃]ᵢ increase that evoked a [Ca²⁺]ᵢ increase and a later cellular response, the CBF increase.

Activation of the PLC pathway is known to evoke the production of diacylglycerol, the activator of PKC (30). Therefore, three experimental approaches were used to examine the participation of PKC in the [Ca²⁺]ᵢ increase. First, ciliated cells were preincubated with a PKC inhibitor, GF-109203X (41). Figure 3E shows that in the presence of 2 µM GF-109203X, there was a reduction of 54.0 ± 3.8% in the plateau value compared with the control response. However, the change in the peak value (14.5 ± 2.7%) was not statistically significant. Second, cells were stimulated by ATP in the presence of 500 nM PMA, a PKC activator (33). As shown in Fig. 3F, PMA did not evoke any change in basal [Ca²⁺]ᵢ. These results demonstrate that activation of PKC did not bring about an increase in [Ca²⁺]ᵢ, but that activation of this pathway is necessary to produce the ATP response resulting in [Ca²⁺]ᵢ influx. Furthermore, PKC was not involved in the [IP₃]ᵢ increase induced by ATP because preincubation with GF-109203X or PMA evoked no change in IP₃ production (data not shown). In addition, we observed a reduction of 78.5 ± 3.6% in the increase in CBF induced by ATP when ciliated cells were preincubated with 2 µM GF-109203X, and we observed a transient CBF increase (maximum of 61.2 ± 2.9%) after PMA treatment (28). Figure 3F shows that coincubation with 100 µM ATP and 500 nM PMA produced a plateau value 1.8-fold higher than the control response; however, this coapplication induced no change in the peak value. Third, we quantified PKC activity in oviductal ciliated cells stimulated with ATP. Figure 3G shows the time course of the effect of 100 µM ATP on PKC activity; there was a peak at 60 s that correlated with the appearance of the plateau component. The effect of ATP on PKC activity was dependent on ATP concentration (Fig. 3H), with an EC₅₀ of 9.3 ± 0.2 µM. Because PKA has been implicated in the control of CBF through a release of Ca²⁺ from intracellular stores (4), we quantified PKA activity in oviductal ciliated cells. Figure 3G shows that PKA was not involved in the ATP transduction pathways. Taken together, these results suggest that PKC is a positive modulator of the Ca²⁺ influx and that PKC is probably a second messenger downstream of [Ca²⁺]ᵢ in the ATP effect.

Localization of IP₃ receptors in oviductal ciliated cells. On the one hand, we report that IP₃ receptors are present in ciliated cells and that they participate in the transduction pathways induced by ATP; on the other hand, it was previously suggested that multiple IP₃ receptor intracellular locations could be related to the differing roles of these receptors in Ca²⁺ mobilization (9, 34). To determine the location of IP₃ receptor types 1 and 3 in oviductal ciliated cells, immunofluorescence and confocal microscopy were performed with cultured ciliated cells using anti-IP₃ receptor types 1 and 3 antibodies (Fig. 4). Figure 4A is a photomicrograph of red fluorescence indicating fluorescence was detected in...
ciliated cells (data not shown). Interestingly, when the two receptor types were visualized in the same cell (Fig. 4, E–H), the type 3 receptor (red fluorescence in Fig. 4E) was concentrated at the apical pole of the cell, whereas the type 1 receptor (green fluorescence in Fig. 4F) was homogeneously distributed. To extend our observation, we performed immunogold electron microscopy to determine the subcellular location of IP3 receptor types 1 and 3 in oviductal ciliated cells (Fig. 5).
whereas practically no gold particles were found in the nuclear and plasma membranes (see Fig. 5C, solid bar). IP$_3$ receptor type 3-reacting gold particles were also found in the nucleus and the endoplasmic reticulum of oviductal ciliated cells; however, in contrast to IP$_3$ receptor type 1, type 3-reacting gold particles were found in the nuclear and plasma membranes (see Fig. 5C, shaded bar). Representative photomicrographs of the subcellular localization of IP$_3$ receptor type 3-reacting gold particles were found in the nuclear and plasma membranes.

Fig. 4. Localization of IP$_3$ receptor types 1 and 3 in oviductal ciliated cells. A: immunofluorescence localization of IP$_3$ receptor type 3 in cultured ciliated cells. B: ciliated cells shown in phase-contrast image. C: immunofluorescence localization of the IP$_3$ receptor type 1 in cultured ciliated cells. D: ciliated cells shown in phase-contrast image. E and F: simultaneous detection by immunofluorescence of both IP$_3$ receptor types 3 and 1 in the same ciliated cell. G and H: ciliated cell in phase-contrast image (G) and merged fluorescence image (H).
particles in plasma membrane (Fig. 5A) and nucleus (Fig. 5B) are shown. In parallel, control experiments without primary antibody demonstrated that no gold particles were found in identical ciliated tissues (data not shown). This evidence demonstrates a diverse intracellular distribution of the IP3 receptor types 1 and 3 in ciliated cells, which could be related to the \([\text{Ca}^{2+}]_i\) increase induced by ATP-dependent IP3 receptor activation from several \([\text{Ca}^{2+}]_i\) stores, including the nucleus, the endoplasmic reticulum, and the extracellular medium.

**Participation of the CaM pathway in the ATP effect in ciliary activity.** Because of the \([\text{Ca}^{2+}]_i\) increase induced by ATP, which is responsible for the increment in CBF, we anticipated the participation of CaM, a known calcium-dependent protein, in the control of ciliary activity (52). We therefore asked whether this protein is capable of modifying the \([\text{Ca}^{2+}]_i\) mobilization induced by ATP. Figure 6A shows that preincubation with 50 \(\mu\text{M}\) W-7, a CaM inhibitor, brought about 78.2 \(\pm\) 5.2\% inhibition of the maximum CBF induced by 100 \(\mu\text{M}\) ATP. In addition, Fig. 6C shows that preincubation with 50 \(\mu\text{M}\) W-7 caused only a small, transient increase in \([\text{Ca}^{2+}]_i\) in response to ATP. To determine whether CaMKII, a CaM activity-dependent kinase (53), is involved in the transduction pathway activated by ATP, we preincubated the ciliated cells for 15 min (through hyposmotic shock) with 30 \(\mu\text{M}\) CBP, a synthetic peptide that inhibits CaMKII (49) by blocking CaM activation. After this preincubation, the solution was replaced by control Hanks’ buffer for 10 min before application of 100

![Fig. 5. Subcellular localization of IP3 receptor types 1 and 3 in oviductal ciliated cells. A and B: representative photomicrographs of sections incubated with anti-IP3 receptor type 3 antibody followed by immunogold electron microscopy. Gold particle labeling is localized to the plasma membrane (PM) (A) and nucleus (N) (B). Bars and arrows indicate scale and gold particles, respectively. C: density of gold particles observed for IP3 receptor type 1 (black bar) and type 3 (gray bar) in N, endoplasmic reticulum (ER), PM, vesicles (V), and nuclear membrane (NM). Data are means \(\pm\) SE \((n = 10)\). Asterisk indicates significant statistical difference in density of gold particles between both types of IP3 receptors.](http://ajpcell.physiology.org/)

![Fig. 6. Participation of \([\text{Ca}^{2+}]_i\)-calmodulin pathway in the effect of ATP on ciliated cells: effect of 50 \(\mu\text{M}\) W-7 and 30 \(\mu\text{M}\) CBP on CBF (A and B) and \([\text{Ca}^{2+}]_i\) (C and D) increases induced by 100 \(\mu\text{M}\) ATP \((n = 12)\). Preincubation with W-7 (A, □) or CBP (B, ○) for 10 min blocked CBF increase induced by ATP (A, ▪, or B, •). Data are means \(\pm\) SE \((n = 10)\). Moreover, preincubation with W-7 (C) or CBP (D) evoked only a small, transient increase in \([\text{Ca}^{2+}]_i\), induced by ATP. Traces correspond to average of 8 responses of individual cells. Bars indicate incubation time with reagents.](http://ajpcell.physiology.org/)

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\( \mu M \) ATP (Fig. 6, B and D). Interestingly, the time course of the CBF response to ATP showed a 74.3 \( \pm \) 3.7% inhibition of the maximum response in the presence of CBP (Fig. 6B), which was very similar to the extent of CaM inhibition (\( P < 0.05 \)). Moreover, the effect of 30 \( \mu M \) CBP also resulted in a small, transient increase in \([Ca^{2+}]\), in the presence of ATP (Fig. 6D). Therefore, a high degree of similarity was found when we compared the time courses of CBP and W-7 effects on \([Ca^{2+}]\), and CBF increases induced by ATP, which suggests that the effect of CaMKII can explain the CaM response temporally and quantitatively because CaMKII is a second messenger downstream of CaM activation. Parallel experiments showed that hyposmotic shock without CBP did not change the response of CBF or \([Ca^{2+}]\), to ATP. On the other hand, the \([Ca^{2+}]\), increase induced by 100 \( \mu M \) ATP was almost completely blocked when ciliated cells were pretreated with 1 \( \mu M \) thapsigargin followed by 30 \( \mu M \) CBP. These results suggest that CaMKII is necessary to produce complete \( Ca^{2+} \) entry involved in the ATP effect on CBF.

**DISCUSSION**

This report demonstrates for the first time the presence and the subcellular localization of \( IP_3 \) receptor types 1 and 3 in ciliated cells. We also experimentally explored the dynamic processes generating the \( IP_3 \) and \([Ca^{2+}]\), increases induced by ATP and their association with the cellular response, an increase in CBF. The complete \([Ca^{2+}]\), response is described by two components: first, a transient peak component dependent on \( IP_3 \) receptor activation, followed by a second component consisting of an elevated plateau dependent on \( Ca^{2+} \) influx, which is mediated by \( IP_3 \) receptor activation. Judging by the similarity in the forms of the time courses of \([IP_3] \) response, \([Ca^{2+}]\), response, PKC activity, and CBF increase, it is likely that the transduction mechanisms are highly coordinated to control the physiological response of ciliary activity.

Xestospongin C almost completely blocked the first component and reduced the second component of the response to ATP. Furthermore, after treatment with both thapsigargin and xestospongin C, the second component of the \([Ca^{2+}]\), increase induced by ATP was also almost completely blocked. These results indicate an interaction between these components through \( IP_3 \) receptor activation-dependent \( Ca^{2+} \) influx. Direct activation of \( Ca^{2+} \) entry by plasma membrane \( IP_3 \) receptors has been characterized electrophysiologically in a variety of cells, including T-lymphocytes (20), olfactory neurons (22), vascular endothelial cells (43), and a carcinoma cell line (17). Moreover, a tight functional interaction between \( IP_3 \) receptors and store-operated channels can regulate capacitative \( Ca^{2+} \) entry (18). This result indicates the direct participation of \( IP_3 \) receptors in the induction of the \( Ca^{2+} \) influx, an idea that is supported by our observation that the \( IP_3 \) receptor type 3 is localized in the plasma membrane of the oviductal ciliated cells. Because the half-life of \( IP_3 \) is 9 \( \pm \) 2 s in single cells (48), and because the generation of the maximum increase in \([IP_3] \) synthesis was transient, an additional increase in \([IP_3] \) synthesis would be necessary to generate the latter \( Ca^{2+} \) increase because of the \( Ca^{2+} \) influx. Significantly, there was a good correlation between the generation of the second \([IP_3] \) peak at 60 s and the \([Ca^{2+}]\), change evoked by \( Ca^{2+} \) influx, an observation that supports the possibility that \( IP_3 \) participates directly in triggering the ATP-dependent \( Ca^{2+} \) influx. Electrophysiological recordings of \( Ca^{2+} \) channels are needed to determine the function of \( IP_3 \) receptors involved in the control of \( Ca^{2+} \) influx in oviductal ciliated cells.

Because contamination of commercial samples of adenosine nucleotides has been reported (23), the \([Ca^{2+}]\), increase could in theory be induced by ADP rather than by ATP, acting via \( P_2Y \) receptors coupled to \( Ca^{2+} \) mobilization. Regarding this concern, we have never observed any change in CBF in the presence of ADP (28). Moreover, ADP did not change \([Ca^{2+}]\), in ciliated cells. Furthermore, the EC\(_{90}\) and maximal effect parameters that describe the concentration-response curve of the \([Ca^{2+}]\), increase induced by ATP were not affected in the presence of ADP (data not shown). Consequently, potential traces of ADP contaminating commercially available ATP should not modify the interpretation of our results.

The \( IP_3 \) receptor types 1 and 3 share a subcellular distribution, with both forms being localized to the nucleus and the endoplasmic reticulum. Although we anticipate that both \( IP_3 \) receptors participate in the \( Ca^{2+} \) response induced by ATP, at this moment there is no evidence for any selectivity of xestospongin C between the \( IP_3 \) receptor types 1 and 3, supporting the possibility that these receptors could induce the \([Ca^{2+}]\), increase necessary to evoke a CBF increase in oviductal ciliated cells. The \( IP_3 \) receptor type 3 (which possesses 5-fold greater affinity for \( IP_3 \) than the type 1 receptor) can be activated before type 1 by a small change in \([IP_3] \), which can increase the \([Ca^{2+}]\), to 100–200 nM (5). This hypothesis is supported by the single-channel differential \( Ca^{2+} \) activation properties that result in an apparent higher in vivo \( IP_3 \) sensitivity of the type 3 receptor under resting levels of \( Ca^{2+} \) compared with type 1 (25). On the other hand, it has been reported that higher concentrations of \([Ca^{2+}]\), (\( \geq 500 \) nM) cause a decrease in the \( IP_3 \) binding to \( IP_3 \) receptor type 1 while increasing binding to the type 3 receptor (5). In contrast to this observation, Mak et al. (25) found a similar inhibition of the activities of \( IP_3 \) receptor types 1 and 3 by \( Ca^{2+} \) in this concentration range. Considering the subcellular distribution of the \( IP_3 \) receptor types 1 and 3, it is possible that the first \( Ca^{2+} \) mobilization originates from the nucleus and the endoplasmic reticulum via activation of the type 3 receptor, after which \( IP_3 \) receptor type 1, also localized in these organelles, participates in \( Ca^{2+} \) mobilization. Later, probably when \([Ca^{2+}]\), has reached 400 nM, the \( IP_3 \) receptors become inactivated by \( Ca^{2+} \) and induce the exponential decay in the response (7). It is important to emphasize that the inactivation of the \( IP_3 \) receptor by \( IP_3 \) can also contribute to the exponential decay in the response without changes in \([Ca^{2+}]\), (24). This exponential decay was unaffected by \( Ca^{2+} \) influx, supporting the idea that \( IP_3 \) receptor type 3 in the plasma membrane could be activated after intracellular \( IP_3 \) receptor types 1 and 3 because of subsequent generation of \( IP_3 \). Taken as a whole, this scenario would explain the increase in \([Ca^{2+}]\); however, experiments to control separately the activation of the two types of \( IP_3 \) receptor associated with \( Ca^{2+} \) mobilization are required to completely understand the molecular mechanism of \( Ca^{2+} \) mobilization in ciliated cells.

We found that PKC activity is stimulated by ATP and is necessary to modulate \( Ca^{2+} \) influx induced by ATP. However, \( Ca^{2+} \) mobilization was not triggered in this process. Furthermore, PKC also has a role downstream of \( Ca^{2+} \) influx because...
this mediator can transiently increase CBF independently (28). On the other hand, a PKC effect upstream of the [IP$_3$] increase can probably be excluded because the PKC activity did not modify the concentration of this mediator in any of the experiments performed in this study.

Our results suggest that the increase in CBF induced by ATP in oviductal ciliated cells requires Ca$^{2+}$-dependent proteins such as CaM and CaMKII. The similarity in the time courses of the effects of CaM and CaMKII inhibitors on [Ca$^{2+}$]$_j$, and CBF increases induced by ATP can be explained by the activation of CaM and the subsequent increase in CaMKII activity. CaMKII may target the IP$_3$ receptor type 3, because these proteins have been found to be colocalized in the apical domain in gastrointestinal tissues and the IP$_3$ receptor type 3 has potential phosphorylation sites for CaMKII (26). On the other hand, an effect upstream of the [IP$_3$] increase induced by ATP is probably unlikely because changes in CaM and CaMKII activities did not affect the concentration of [IP$_3$] in our experiments.

In summary, this study was focused on the elucidation of the relationship between the subcellular distribution and the functional effects of the IP$_3$ receptors, which we postulate are associated with the ATP transduction pathway. Further experiments examining the ATP transduction pathway in oviductal ciliated cells are required to understand completely the role of IP$_3$ receptors in the control of [Ca$^{2+}$]$_j$, in these cells.

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