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ATP and adenosine trigger the interaction of plasma membrane IP_3 receptors with protein kinase A in oviductal ciliated cells

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Abstract

We have demonstrated that adenosine did not produce any change of intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$) in oviductal ciliated cells; however, it increased the ATP-induced Ca^{2+} influx through the activation of protein kinase A (PKA). Uncaging of IP_3 and cAMP triggered a larger Ca^{2+} influx than did IP_3 alone. Furthermore, the IP_3 effect was abolished by Xestospongin C, an IP_3 receptor blocker. Whole-cell recordings demonstrated the presence of an ATP-induced Ca^{2+} current, and the addition of adenosine increased the peak of this current. This effect was not observed in the presence of H-89, a PKA inhibitor. Using excised macro-patches of plasma membrane, IP_3 generated a current, which was higher in the presence of the catalytic PKA subunit and this current was blocked by Xestospongin C. We show here that activation of plasma membrane IP_3 receptors directly triggers Ca^{2+} influx in response to ATP and that these receptors are modulated by adenosine-activated PKA.

Keywords: Signal transduction; Ciliated cells; Synergism; IP_3 receptors; Calcium; ATP; Adenosine

Control of ciliary beat frequency (CBF) is responsible for the regulation of mucociliary transport and transport of gametes and embryos [1]. In oviductal ciliated cells, the activation of interacting ATP and adenosine transduction pathways produce oscillations in the magnitude of synergistic increase of CBF [2,3]. It has been suggested that the synergism of CBF induced by ATP and adenosine [2,4] depends on the changes of intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$) [4], and it has been widely shown that a Ca^{2+} release from intracellular stores results in a CBF increase [4–8]. Furthermore, activation of IP_3 receptors are known to mediate this response [9–11]. Recently we have shown a different subcellular localization of the IP_3 receptor types 1 and 3 in cultured ciliated cells. The IP_3 receptor type 1 was found exclusively in the endoplasmic reticulum and nucleus; in contrast, the type 3 was also found in the plasma membrane [9]. Further, we have shown a bimodal increase of the $[IP_3]$ in oviductal ciliated cells with peaks strongly correlated to Ca^{2+} release from intracellular stores and Ca^{2+} influx [9]. Any occurrence of activation of IP_3-evoked currents through plasma membrane IP_3 receptors was, for some time, unclear [12], but a recent report suggests that the presence of a few IP_3 receptors expressed at the plasma membrane can induce a substantial Ca^{2+} entry in both B-cells and DT40 cells [13].

We have shown that ATP activates the $P2Y_2$ receptor in the plasma membrane, which in turn activates the PKC, IP_3 production and plasma and intracellular membrane IP_3 receptors [4,9]. These events trigger an increase of $[Ca^{2+}]i$ and activation of CaMKII. In a parallel pathway, adenosine activates the A_{2a} receptor, cAMP production and PKA [4,14]. Phosphorylation of the components of the ciliary machinery could then regulate ciliary activity [15–17]. Also, it has been reported that PKA can modulate the activity of IP_3 receptors [18,19]. We hypothesized that IP_3 receptors are target of the activation of adenosine transduction pathways.

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We show that ATP triggers a Ca^{2+} influx through activation of plasma membrane IP_3 receptors. In addition, these receptors are phosphorylated by adenosine-activated PKA to generate the synergistic Ca^{2+} influx.

Materials and methods

Measurement of [Ca^{2+}]i. [Ca^{2+}]i was determined using a spectrofluorometric technique described previously [20]. Primary cell cultures from hamster oviductal epithelium [5,21] exhibiting spontaneous ciliary activity at 37 °C were loaded with 2 μM Fura 2-AM for 30 min. The intensity ratio (349/380 nm) for single cells was continuously recorded and cultures were superfused with solutions containing the drugs to be tested [9].

Intracellular uncaging of IP_3 and cAMP. Ciliated cells were incubated 30 min at 34 °C in a hypo-osmotic Hank's solution (35% reduction of normal osmolality, 290 mOsm), containing 2 μM FURA 2-AM, 50 μM DMNB-cAMP and 50 μM NPE-IP_3. Uncaging of IP_3 and cAMP was triggered by 2 ms UV light at 264 and 325 nm, respectively. [Ca^{2+}]i was calculated as above.

Electrophysiological recording of ATP and IP_3-triggered currents in cultured ciliated cells. Cultured ciliated cells were incubated in an extracellular solution containing 120 mM NaCl, 2.5 mM KCl, 4 mM $CaCl_2$, 5 mM HEPES, 5 mM glucose, pH 7.4. Recordings were performed using borosilicate glass micropipettes (resistance 20–25 MΩ when filled with a standard intracellular solution, 10 mM NaCl, 0.1 mM $CaCl_2$, 125 mM KCl, 10 mM EGTA, 5 mM HEPES; pH 7.4). ATP-triggered currents were recorded using whole-cell voltage clamp configuration (pCLAMP 8.0, Axopatch 1D, Axon Instruments), filtered at 5 kHz and holding potential = −80 mV.

Inside-out macro-patches from cultured ciliated cells were used for recording of IP_3-triggered current. Bath solution contained 140 mM KCl, 10 mM HEPES, 0.5 mM Na_2ATP, 200 μM BAPTA, pH 7.4 and glass micropipettes of 0.2–0.4 MΩ were used. Micropipettes solution was identical, but without Na_2ATP, to maintain symmetrical conditions [22]. Currents were induced by rectangle voltage pulses [23] from −40 mV to 40 mV in the presence of 30 μM IP_3 with or without 1 mU catalytic PKA subunit.

Data analysis. Statistical comparisons between different experimental conditions were made using ANOVA (significant difference of $P < 0.05$).

Results

We have shown that ATP induces an increase of [Ca^{2+}]i [9] and suggested that Ca^{2+} could be the target of 'cross-talk' between ATP and adenosine in ciliated cells [4]. To test this, we evaluated the role of the adenosine transduction pathway in the ATP-dependent Ca^{2+} response. The ATP-dependent-[Ca^{2+}]i increase was statistically larger in the presence of adenosine (Fig. 1A, the response to 100 μM ATP, integrated over time, is 24719 ± 960 nM s, $n = 12$, and to ATP + 1 μM adenosine is 63679 ± 1455 nM s, $n = 8$, $P < 0.05$, and Table 1). The decay of the [Ca^{2+}]i response to ATP can be fitted by an exponential equation where parameters such as peak, decay constant and plateau can be extracted [9]. Peak values did not change in the presence of 1 μM adenosine (Fig. 1A and Table 1) or 250 μM 8-Br cAMP (a membrane permeant PKA activator; data not shown). Furthermore, the adenosine effect is abolished in ciliated cells pre-incubated with H-89, PKA inhibitor [24] (compare plateau values between green and red curves in Fig. 1A, Table 1). The ATP response can be also modulated by threshold concentrations of adenosine [4], but interestingly, the magnitude of their effect is increased (Fig. 1C and D), which is in agreement with a higher 'capacity of interaction' observed at low concentration of agonist [3]. Fig. 1B shows no difference in the peak value of the [Ca^{2+}]i response to ATP, ATP + 1 μM adenosine or ATP + 100 nM adenosine. However, the response to ATP integrated over time and the plateau values does show an increase in the presence of either adenosine concentration (Fig. 1C and D). These data strongly suggest that only the sustained component, associated with the Ca^{2+} influx [9], was modulated by the adenosine transduction pathway-activated PKA (Table 1). We have recently demonstrated that both the transient intracellular store-dependent Ca^{2+} release, and the sustained component are dependent upon increasing the [IP_3] and on IP_3 receptor activation [9], and we also demonstrated that the IP_3 receptor type 3 is located in the plasma membrane of ciliated cells [9]. To clarify the situation we used caged IP_3 and cAMP to determine their specific effects on [Ca^{2+}]i. IP_3 triggered a transient increase of [Ca^{2+}]i (Fig. 2A, black arrows) with a response of 720 ± 53 nM s. In contrast, cAMP did not produce any change of [Ca^{2+}]i (gray arrows). As might be expected, the application of Xestospongin C, a specific IP_3 receptor blocker [25], blocked completely the IP_3 effect (Fig. 2B). Fast sequential uncaging of IP_3 and cAMP triggered a larger Ca^{2+} increase than did IP_3 alone, with a response of 2100 ± 105 nM s ($P < 0.05$, $n = 8$, Fig. 2C), together with oscillations in [Ca^{2+}]i. Extracellular EGTA significantly reduced the Ca^{2+} increase induced by IP_3 to 340 ± 23 nM s ($P < 0.05$, $n = 8$, Fig. 2D). Under these conditions cAMP did not have any effect on IP_3-induced release of Ca^{2+} (350 ± 18 nM s). Pre-incubation with thapsigargin, a Ca^{2+} pump ATPase blocker that reduces the CBF increase induced by ATP [4], also significantly reduced the Ca^{2+} increase induced by IP_3 (368 ± 15 nM s), but did not prevent the higher Ca^{2+} influx generated by the uncaging of IP_3 and cAMP (930 ± 36 nM s, Fig. 2E). As would be expected, co-application of thapsigargin and EGTA completely blocked the IP_3 effect on [Ca^{2+}]i (Fig. 2F). This suggests that cAMP only affects the IP_3 receptor-activated Ca^{2+} influx. On the other hand, it has been postulated that the Ca^{2+} influx controls the CBF in rat ciliated ependymal cells through activation of CRAC channels [26]. However, in our study, pre-incubation with 50 μM SKF-96365, a CRAC channel blocker, did not affect the [Ca^{2+}]i response to ATP in oviductal ciliated cells (compare black curves in Fig. 1A and E). In another report, TRPC channels have been shown to trigger Ca^{2+} influx through activation by DAG [27], which is a second messenger in the ATP transduction pathway in oviductal ciliated cells [9,14]. Preliminary results show that 300 μM OAG, a DAG analogue, did not change the basal [Ca^{2+}]i or the [Ca^{2+}]i response to ATP (data not shown). Taken together, these results indicate that the IP_3 receptors located in the plasma membrane are a target of adenosine transduction pathways.

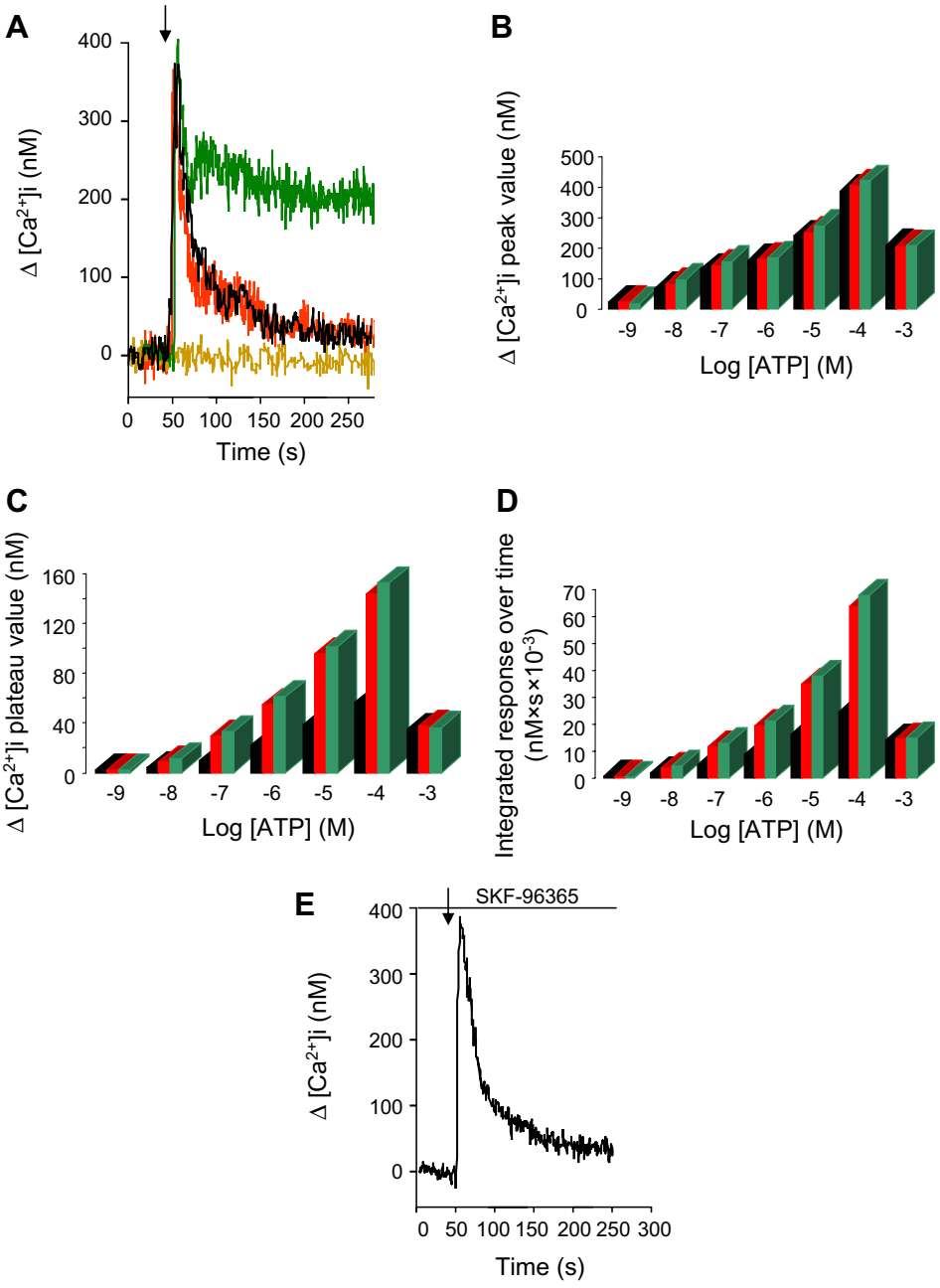

Fig. 1. Synergism of the ATP-dependent Ca^{2+} mobilization induced by the adenosine transduction pathway. (A) Co-application of 1 μM adenosine and 100 μM ATP (green line) generates a higher Ca^{2+} mobilization particularly on the plateau of the ATP response (black line), which is associated to Ca^{2+} influx. Pre-incubation with 1 μM H-89 blocks the adenosine effect on ATP response (red line). Application of adenosine does not change $[Ca^{2+}]i$ in ciliated cells (yellow line). Representative traces of 10 experiments. Application of ATP and/or adenosine is indicated by an arrow. (B–D) Concentration-response curves of the peak (B), plateau (C) and integral (D) values, induced by ATP (black columns), ATP + 1 μM adenosine (red columns), and ATP + 100 nM adenosine (green columns). (E) Fifty micromolar of SKF-96365 does not modify the ATP-dependent $[Ca^{2+}]i$ increase. Application of ATP is indicated by an arrow. Representative trace of 6 experiments.

ATP triggered a Ca^{2+} current in ciliated cells using whole-cell patch clamp recordings (Fig. 3A), which was blocked in the presence of Xestospongin C (compare black and pink lines) or EGTA (Fig. 3B, gray line). In addition, no voltage-dependent currents were observed in ciliated cells (Fig. 3F). Furthermore, adenosine did not evoke currents in ciliated cells (data not shown). Co-application of ATP and adenosine generated a synergistic Ca^{2+} current (green line, Fig. 3B) and pre-incubation with H-89 prevented the adenosine effect on Ca^{2+} influx (red line)—effectively returning the response to that seen in the presence of ATP alone. As expected, ATP treatment revealed almost identical curves of normalized current and $[Ca^{2+}]i$ increase (Fig. 3C). In order to identify a current activated directly by IP_3, we performed inside-out macro-patches to increase the number of IP_3 receptors analyzed. The application of

Table 1
Adenosine effect on the Ca^{2+} response induced by ATP

Treatment	Peak value (nM)	Plateau value (nM)	Decay constant (s)	Integrated response over time (nM s)	*n*
100 μM ATP (control)	380 ± 28	58 ± 8	22 ± 2	24719 ± 960	12
100 μM ATP + 1 μM adenosine	404 ± 33	143 ± 5*	80 ± 3*	63679 ± 1455*	8
100 μM ATP + 1 μM adenosine + 1 μM H-89	374 ± 36	68 ± 6	28 ± 3	24133 ± 868	7

Data are expressed as means ± SEM.
* Indicates significant difference between control and treated groups ($P < 0.05$).

IP_3 generated a current, which was higher in the presence of the catalytic PKA subunit (compare black and green line, Fig. 3D). Pre-incubation with Xestospongin C blocked both effects completely (pink line, Fig. 3D). The *I–V* curve shows that PKA significantly increased ($P < 0.05$, $n = 12$) the conductance of IP_3 receptors, from 11.7 ± 1.3 nS to 18.2 ± 1.8 nS (Fig. 3E). These results suggest that the synergism starts at early steps on ATP transduction pathways, where IP_3 receptors located in plasma membrane are phosphorylated by PKA following activation of adenosine transduction pathway.

Discussion

We demonstrate that the activation of the adenosine transduction pathway results in an enhanced Ca^{2+} influx triggered by either ATP or IP_3. Increments of $[Ca^{2+}]i$ response after co-stimulation of each transduction pathway were very similar, ~2.6-fold for ATP and adenosine, and ~ 2.9-fold for IP_3 and cAMP together. Besides, EGTA or Xestospongin C but not thapsigargin blocked the synergistic increase of $[Ca^{2+}]i$ induced by IP_3 and cAMP. Taken together, these evidences indicate that no difference on the signal amplification was present at this level of transduction and that the synergism should therefore start upon Ca^{2+} influx, itself activated by plasma membrane IP_3 receptors [9]. We also explored alternative pathways known to be involved in triggering Ca^{2+} influx [26,27]. Oviductal ciliated cells did not show activity of voltage-dependent channels, nor CRAC or TRPC channels, which strongly support our evidence of plasma membrane IP_3 receptors acting as direct mediators of Ca^{2+} influx.

Although Ca^{2+} influx is not necessary to trigger the CBF increase in the ATP transduction pathway, it maintains and amplifies the response [4]. Furthermore, the Ca^{2+} current triggered by ATP followed the same response-concentration pattern as that seen in the Ca^{2+} influx component, and adenosine alone did not evoke an increase of $[Ca^{2+}]i$ and of electrical activity in ciliated cells. This might suggest that synergism plays a supporting rather than triggering role in the steps of these transduction pathways.

It has been proposed that one of the mechanisms of 'cross-talk' between Ca^{2+} and cAMP signaling depends upon phosphorylation of intracellular IP_3 receptor types 1 [18,19,28,29] and 3 [18,19] by PKA. We demonstrate here that activation of plasma membrane IP_3 receptors on inside-out macro-patches generated a conductance almost a 65% higher after application of the catalytic PKA subunit. It is known that the unitary conductance of IP_3 receptors is approximately 370 pS, and the open probability about 0.5 [23] at a free $[Ca^{2+}]$ of 220 nM (identical to our experiments). Hence a number of 60–65 IP_3 receptors are expected to be present on the plasma membrane macro-patches of ciliated cells studied here. Furthermore, using immunogold electron microscopy we demonstrated the presence of approximately 20 IP_3 receptors in 1 μm^2 of plasma membrane [9] in a non-homogenous distribution, which also supports a patch arrangement of receptors. Recent results suggest that clusters of 50–70 IP_3 receptors are responsible for intracellular Ca^{2+} signaling through the endoplasmic reticulum [30]. This is supported by the absence of single channel currents induced by IP_3 in ciliated cells with micropipettes of 15 MΩ or higher. In accordance with this observation, Dellis et al. [13] did not detect IP_3-gated currents in isolated inside-out plasma membrane patches. However, using perforated-patch and whole-cell recordings they were able to identify only 2–3 IP_3 active channels per cell (B and DT40 cells), which triggered a substantial Ca^{2+} entry. The number of IP_3 receptors expressed at the plasma membrane could be associated with different mechanisms of Ca^{2+} mobilization [12]. Ciliated cells require a considerable consumption of kinetic energy to maintain the basal CBF [2,31], which is increased 4-fold in the presence of ATP and adenosine [2]. As plasma membrane IP_3 receptors are the early effectors in the ATP transduction pathways, the simultaneous activation of several hundred channels could facilitate the downstream effect and trigger a fast response. Also, to maintain a coordinated ciliary activity in the oviductal epithelium, gap junctions [32] might play an important role in spreading intracellular Ca^{2+} waves across the epithelium, inducing a paracrine Ca^{2+} increase in adjacent cells. Consequently we suggest that (i) a high number of IP_3 receptors in ciliated cells is necessary to facilitate the energy-$[Ca^{2+}]i$ relationship and (ii) clustered distribution of receptors can maximize the effect on ciliary machinery. We show that the synergism begins at clustered-IP_3 receptors located in plasma membrane that are phosphorylated by PKA. Although PKA increases the activity of intracellular IP_3 receptors type 3 through changes in the kinetic properties of the channel [18,19];

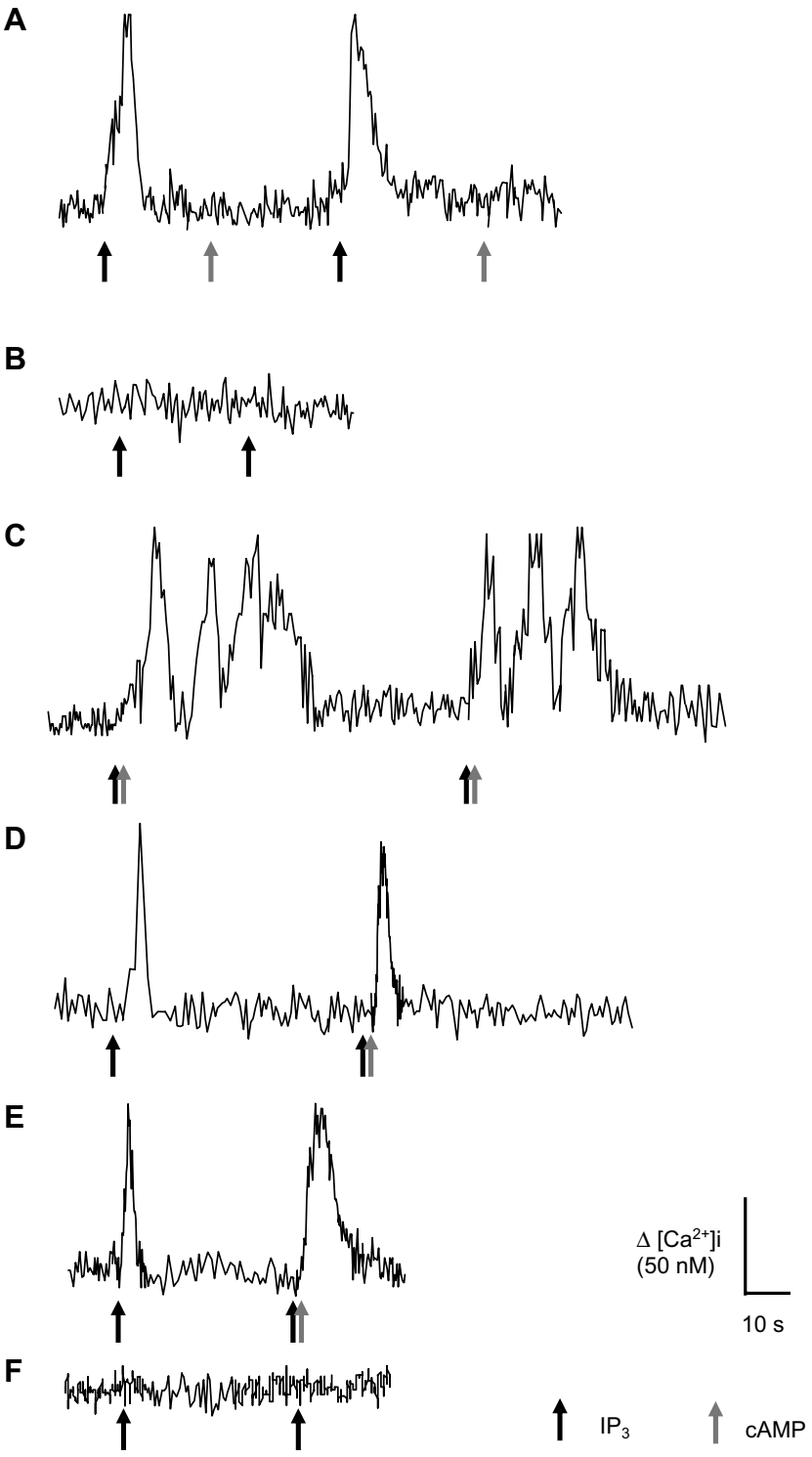

Fig. 2. Effect of cAMP on the Ca^{2+} response induced by IP_3. (A) Uncaging of IP_3 (black arrow) but not cAMP (gray arrow) triggers a transient $[Ca^{2+}]i$ increase. Representative traces of 8 experiments. (B) Pre-incubation with 5 μM Xestospongin C completely inhibits the IP_3 effect on Ca^{2+} mobilization ($n = 5$). (C) Co-uncaging of IP_3 and cAMP induced a synergistic $[Ca^{2+}]i$ increase in comparison to IP_3 alone ($P < 0.05$, $n = 8$). (D) The presence of EGTA reduces the $[Ca^{2+}]i$ increase triggered by IP_3 and prevents the synergistic effect of cAMP. (E) Pre-incubation with 1 μM Thapsigargin also reduces the IP_3-dependent Ca^{2+} mobilization, but the synergistic effect of cAMP is observed. (F) Pre-incubation with Thapsigargin and EGTA completely inhibits the $[Ca^{2+}]i$ increase induced by IP_3. A scale bar for panels (A–F) is shown in figure.

the localization of clustered-IP_3 receptors type 3 in the plasma membrane of ciliated cells [9] could also involve a PKA-dependent-increase in the number of channels activated to facilitate the synergistic effect.

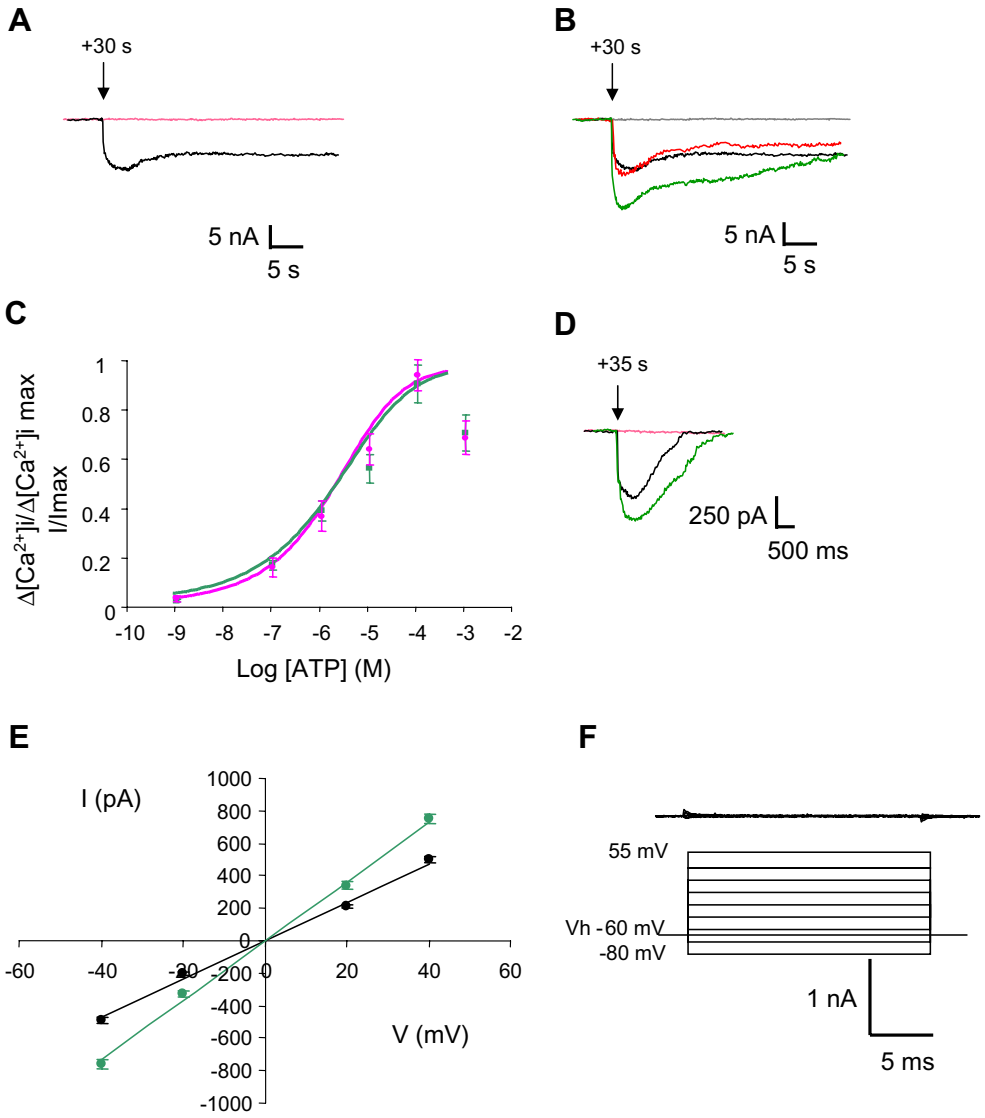

Fig. 3. Synergism of the IP_3 receptor triggered-current. (A) In whole-cell recording, 100 μM ATP induced an inward current (black line), which is blocked when ciliated cells are pre-incubated with 5 μM Xestospongin C (pink line). Representative traces of 8 experiments. Arrow indicates the time after ATP stimulation. (B) Co-stimulation of ciliated cells with 100 μM ATP and 1 μM adenosine (green line) induces a higher inward current in comparison to control (black line). However, the presence of 1 μM H-89 blocked the synergistic effect of adenosine on ATP-dependent inward current (red line). EGTA completely blocks the inward current activated by ATP (gray line). Representative traces of 8 experiments. Arrow indicates the time after ATP stimulation. (C) Normalization of concentration-response curves of inward currents (green squares) and plateau values of $[Ca^{2+}]i$ increase (pink circles) induced by ATP. Solid lines represent sigmoid fitting of the data. Data are shown as means ± SEM. No significant differences are observed between both sets of data ($P > 0.05$, $n = 15$). (D) In inside-out macro-patches of plasma membrane of ciliated cells, 30 μM IP_3 induces a current (black line), which is higher in the presence of 1 mU catalytic PKA subunit (green line). The IP_3-activated current is blocked in the presence of 5 μM Xestospongin C in the bath solution (pink line). Representative traces of 7 experiments. Arrow indicates the time after IP_3 stimulation. (E) *I–V* curves of the IP_3 receptor-activated current in the absence (black circles) or presence of 1 mU catalytic PKA subunit (green circles). Solid lines represent linear regression of data (*r* values for both lines >0.995). (F) Application of rectangular voltage pulses on whole-cell recordings in oviductal ciliated cells. Representative traces of 11 experiments. Vh indicates the holding potential.

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