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Protein kinase A modulates an endogenous calcium channel, but not the calcium-activated chloride channel, in *Xenopus* oocytes

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In *Xenopus* oocytes, Ca^{2+} influx through an endogenous voltage-gated Ca^{2+} channel activates a transient outward Cl^- current ($I_{Cl(Ca)}$), which is potentiated by cAMP increase. The site of cAMP effect appears to be the Ca^{2+} channel instead of the Ca^{2+}-activated Cl^- channel, because cAMP potentiates the Ba^{2+} current through the Ca^{2+} channel in a similar way to the $I_{Cl(Ca)}$, and cAMP does not potentiate the Ca^{2+}-dependent Cl^- current in cells treated with Ca^{2+} ionophore. Using the catalytic subunit of protein kinase A (PKA) and PKA inhibitors, it was shown that PKA is both necessary and sufficient for the cAMP effect on $I_{Cl(Ca)}$. Furthermore, the cAMP/PKA-mediated potentiation of $I_{Cl(Ca)}$ was inhibited by both type 1 and type 2A protein phosphatases.

Protein kinase A; Phosphatase; Calcium channel; *Xenopus* oocyte; Calcium ionophore

1. INTRODUCTION

The membrane of *Xenopus* oocytes contains a voltage-dependent, transient outward current. It is activated by membrane depolarization from the resting potential to a potential more positive than −20 mV and is inactivated at potentials more positive than +50 mV, with the peak activation at between +10 and +20 mV. This current, designated $I_{Cl(Ca)}$, has been shown [1,2] to result from the sequential opening of two ion channels: a voltage-gated Ca^{2+} channel and a Ca^{2+}-dependent Cl^- channel, both of which are endogenous to the membrane of *Xenopus* oocytes. $I_{Cl(Ca)}$ can be potentiated by intracellular injection of cAMP [2,3], implicating a role by either cAMP alone or the cAMP-dependent protein kinase (protein kinase A, PKA). The molecular mechanism underlying cAMP potentiation of $I_{Cl(Ca)}$, however, is not clear, especially with respect to whether the modulation is phosphorylation-mediated and whether both the Ca^{2+} and Cl^- channels are modulated.

Protein kinase modulation of both Ca^{2+} and Cl^- channels via phosphorylation is a common mechanism of regulating membrane currents [4,5]. Ca^{2+} channels mediating the contraction of muscle fibers and exocytosis are modulated by cAMP-dependent phosphorylation. Stimulation of β-adrenergic receptor in the heart leads to an increase in the current of L-type Ca^{2+} channel through PKA phosphorylation [6]. PKA also leads to increase in the currents of Ca^{2+} channels in the brain [7].

Protein phosphorylation also regulates Cl^- channels. Cystic fibrosis transmembrane conductance regulator (CFTR) is a Cl^- channel, and it is gated by a number of mechanisms including protein kinase phosphorylation [8]. Certain mutations in CFTR lead to a loss of modulation by PKA and result in cystic fibrosis, a disease characterized by deficits in salt secretion and the production of thick mucus in airway passages [9]. PKA-dependent phosphorylation has also been found to activate a Cl^- channel in guinea pig ventricles [10]. The activation of this Cl^- current by phosphorylation is involved in the repolarization of the myocyte action potential.

In this study we have asked the question whether the cAMP response results from direct activation of either the Ca^{2+} channel or the Cl^- channel by cAMP or is mediated through PKA. We also asked the question whether the modulation occurs by directly modulating the $I_{Cl(Ca)}$ current in the oocyte membrane or at another effector site. We report here that PKA is the mediator of cAMP effect and that PKA-dependent phosphorylation is both necessary and sufficient for the modulation of $I_{Cl(Ca)}$ current. We also show that the modulation of $I_{Cl(Ca)}$ by PKA is at the Ca^{2+} channel, but not the Cl^- channel.

2. MATERIALS AND METHODS

2.1. *Chemicals and reagents*

Chemicals and L-15 medium were purchased from Sigma. The native catalytic subunit of PKA was purified from bovine heart as described [11]. The PKI peptide was synthesized by the Peptide and Oligonucleotide Synthesis Facility of the Howard Hughes Medical Institute, University of Washington. Phosphatase type 1 and 2A catalytic subunits were purified by published procedures [12].

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2.2. *Preparation of mutant RII*

The mutant RII was generated by substitution of a 20-residue sequence at the position 79–98 of the wild type RII of PKA with a 20-residue sequence of PKI peptide (TTYADFIASGRTGRRNAIHD). The expression vector was constructed using site-directed mutagenesis [13]. A *SalI–HindIII* DNA fragment containing the coding sequence of the hinge region of the wild type RII was ligated into M13 vector as a template. Two unique restriction sites were introduced to flank the sequence encoding residues 79–98 of RII, and were used to remove this sequence. A 60-bp synthetic oligonucleotide with cohesive ends which encodes the 20-residue PKI peptide was ligated to the vector, and the two restriction sites flanking the insert were changed back to the corresponding sequence of the wild type RII by mutagenesis. The *SalI–HindIII* DNA fragment containing the mutation was ligated with the rest of RII coding sequence to create the mutant RII. DNA sequence analysis was performed to verify the mutation region. The recombinant mutant RII was expressed and purified as described [14].

2.3. *Electrophysiology*

Oocyte preparation and two-electrode voltage-clamp recording were described [15]. Injection of cAMP and other solutions was performed with a Drummond microinjector. All injection solutions were diluted in 10 mM Tris (pH 7.4), and the injection volume was 50 nl/oocyte. Oocytes were bathed in the Ringer solution [15] with 6 mM $CaCl_2$ for recording membrane currents, unless specified otherwise. Ba^{2+} currents were measured in 100 mM $BaCl_2$ and 5 mM HEPES, pH 7.6. I_{Ba} was calculated by subtracting the peak inward current in the presence of Cd^{2+} from that in the absence [16].

2.4. *Calcium ionophore experiments*

Oocytes were injected with cAMP (5 pmol/cell) or the same volume of 10 mM Tris (pH 7.4) as control, and were treated for 20–30 min with Ca^{2+}-free Ringer solution containing 5 μM of ionophore A23187 and 1 mM EGTA. Individual oocytes were voltage clamped at −60 mV and superfused with the same solution. Ca^{2+}-activated Cl^- current was recorded by switching the superfusate to Ringer solution containing 6 mM of $CaCl_2$.

3. RESULTS

3.1. $I_{Cl(Ca)}$ is mediated by Ca^{2+} influx and potentiated by cAMP

In defolliculated *Xenopus* oocytes, depolarization of the membrane potential to +20 mV produced both a transient outward current that inactivates in a few seconds and a stable outward current (Fig. 1A). The transient current, termed $I_{Cl(Ca)}$ [1,17], could be derived by subtracting the steady-state value from the peak current (Fig. 1A). $I_{Cl(Ca)}$ was blocked by extracellular application of 100 μM Cd^{2+} (Fig. 1A, bottom panel), indicating its dependence on an influx of extracellular Ca^{2+} through Ca^{2+} channels. Intracellular injection of EGTA (0.4 nmol) also blocked $I_{Cl(Ca)}$ (data not shown), suggesting that the action of Ca^{2+} is from the cytoplasmic side of the membrane.

It has been reported [1,17] that the amplitude of $I_{Cl(Ca)}$ is dependent on membrane potential, with the maximum current occurring at +10 to +20 mV. Fig. 1B shows the current–voltage relationship of $I_{Cl(Ca)}$ in an oocyte (closed circles), with the activation of $I_{Cl(Ca)}$ peaking at ~ +20 mV. When 3′-5′-cAMP (5 pmol) was injected into the cell, the maximal amplitude of $I_{Cl(Ca)}$ was increased (Fig. 1A), without shifting its voltage dependence (Fig. 1B, open circles). The effect is specific to 3′-5′-cAMP, because injection of either 100 pmol of 2′-3′-cAMP or 100 pmol of AMP had no effect on $I_{Cl(Ca)}$

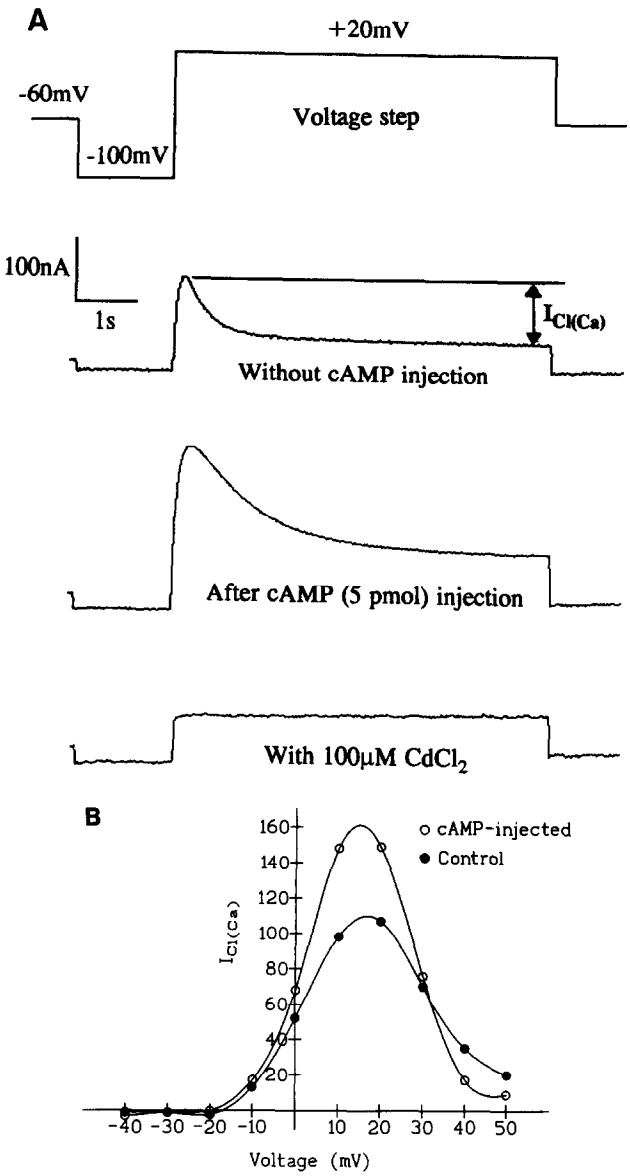

Fig. 1. $I_{Cl(Ca)}$, its dependence on Ca^{2+} influx, and cAMP potentiation. (A) *Xenopus* oocytes were voltage clamped at −60 mV. To activate voltage-dependent currents, the membrane potential was first stepped to −100 mV for 3 s to remove any inactivation and then to +20 mV for 5.5 s (top panel). The resulting currents from an oocyte in Ringer solution with 6 mM Ca^{2+} are shown in the middle, recorded before and after injection of cAMP (5 pmol). $I_{Cl(Ca)}$ was calculated by subtracting the steady-state current from the initial transient peak. In the presence of 100 μM $CdCl_2$, $I_{Cl(Ca)}$ was blocked (bottom panel). (B) Current-voltage relationship of $I_{Cl(Ca)}$ before and after cAMP injection. The membrane potential of an oocyte (in Ringer solution with 6 mM Ca^{2+}) was held at −60 mV, stepped to −100 mV to remove inactivation, and then stepped to various potentials as indicated. $I_{Cl(Ca)}$ was measured before (closed circles) and 5 min after (open circles) injection of cAMP (5 pmol/cell) and plotted against the corresponding membrane potentials. The curves represent polynomial fittings to the data.

(data not shown). 3′-5′-cAMP will henceforth be referred to as cAMP in the rest of this report for simplicity.

The size of $I_{Cl(Ca)}$ is rather consistent in oocytes from the same frog, but may vary by several-fold between batches of oocytes from different frogs (data not shown). The potentiating effect of cAMP on $I_{Cl(Ca)}$, however, is always present. It should be noted that elevated cAMP itself does not *activate* $I_{Cl(Ca)}$ (data not shown); it only *potentiates* $I_{Cl(Ca)}$ when the latter is activated by membrane depolarization.

3.2. *Cyclic AMP potentiation of a Ca^{2+} current contributes to the increase in $I_{Cl(Ca)}$*

Miledi [1] and Barish [17] showed that $I_{Cl(Ca)}$ reflects the sequential activation of two ion channels – a voltage-gated Ca^{2+} channel and a Ca^{2+}-gated Cl^- channel, and that quantitatively Cl^- is the carrier of $I_{Cl(Ca)}$. The presence of the voltage-gated Ca^{2+} channel was shown by Dascal et al. [16] using Ba^{2+} as the charge carrier, because the voltage-dependent Ca^{2+} current is small and is only revealed by Ba^{2+} flux through the Ca^{2+} channel which does not activate the Ca^{2+}-gated Cl^- channel. We sought to characterize the cAMP effect on the Ca^{2+} channel by measuring the current carried by Ba^{2+} through the Ca^{2+} channel.

The net Ba^{2+} current, $I_{(Ba)}$, was obtained by subtracting the membrane current of the oocyte in 100 mM $BaCl_2$ with 100 μM Cd^{2+} from that without Cd^{2+}. $I_{Cl(Ca)}$ was also recorded in the same oocytes for I_{Ba} measurement before and after the cAMP injection in order to compare the quantitative effects of cAMP. As shown in Fig. 2, injection of cAMP enhanced the I_{Ba} by over two-fold (hatched bars). $I_{Cl(Ca)}$ was also enhanced to a similar extent by cAMP injection compared to that of I_{Ba}. The relative extent of potentiation (202% for $I_{Cl(Ca)}$ and 230% for I_{Ba}) was not significantly different from each other ($P > 0.05$, t-test). Because I_{Ba} represents the ion flux through the voltage-gated Ca^{2+} channel, these results suggest that potentiation of $I_{Cl(Ca)}$ by cAMP is associated with a similar increase in this Ca^{2+} current.

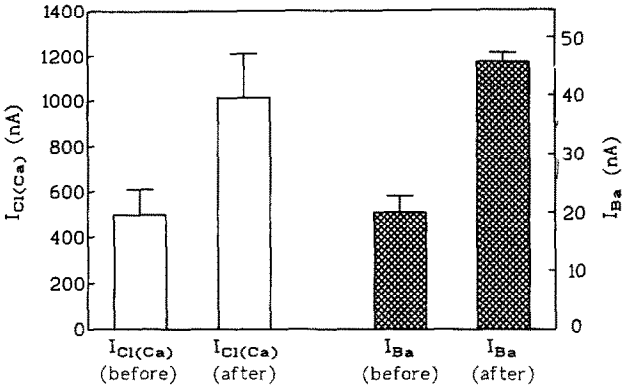

Fig. 2. Effects of cAMP injection on $I_{Cl(Ca)}$ and I_{Ba}. Currents through the voltage-activated Ca^{2+} channel were measured using the Ba^{2+} flux through the Ca^{2+} channel [16] and expressed as I_{Ba}. Voltage steps for activating the Ba^{2+} current were the same as that for $I_{Cl(Ca)}$, and I_{Ba} was obtained by subtracting the peak inward current in the presence of Cd^{2+} from that in the absence. Effects of cAMP on both $I_{Cl(Ca)}$ and I_{Ba} were compared. $I_{Cl(Ca)}$ (open bars) was measured before and 5 min after the cAMP injection (5 pmol/cell, $n = 5$), and I_{Ba} (hatched bars) was measured in the same oocytes before and after the cAMP injection by switching the superfusate bathing the oocytes from the Ringer solution with 6 mM $CaCl_2$ to a solution with 100 mM $BaCl_2$. $I_{Cl(Ca)}$ was calculated as in Fig. 1. I_{Ba} was calculated by subtracting the peak inward current in the presence of Cd^{2+} from that in the absence [16]. Data are presented as the mean of membrane currents with standard error.

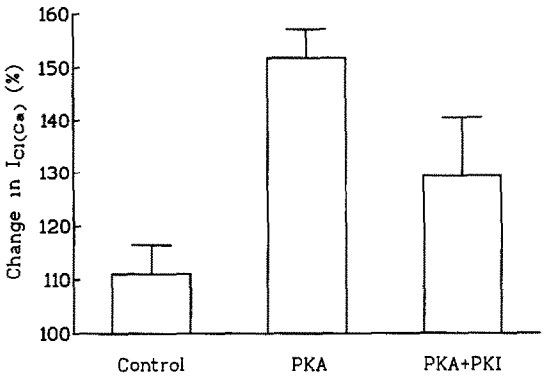

Fig. 3. Enhancement of $I_{Cl(Ca)}$ by PKA and effect of PKA inhibitor PKI. Oocytes were divided into three groups and were injected with 10 mM Tris as control ($n = 9$), the purified catalytic subunit of PKA (70 fmol/cell, $n = 9$), or the PKA catalytic subunit plus PKI peptide (5 pmol/cell, $n = 7$). $I_{Cl(Ca)}$ was recorded from each oocyte before and after the injection. Data are shown as percent change in $I_{Cl(Ca)}$ after injection over $I_{Cl(Ca)}$ before injection (mean ± S.E.M.).

3.3. *The Ca^{2+}-activated Cl^- current is not potentiated by cAMP*

We next examined the cAMP effect on the Cl^- current that is activated by Ca^{2+}. To bypass the Ca^{2+} channel, a Ca^{2+} ionophore, A23187, is employed. Oocytes were incubated with a Ca^{2+}-free Ringer solution containing the Ca^{2+} ionophore, and individual cells were voltage-clamped. Oocytes were superfused with the same Ca^{2+} free Ringer solution, and Ca^{2+}-activated Cl^- currents were elicited by switching the superfusate to a Ringer solution containing 6 mM Ca^{2+}. Since the cell is voltage-clamped at −60 mV without any voltage steps to depolarize the membrane, the endogenous voltage-gated channel should not be open. The membrane current under this condition, then, should represent the current through the Cl^- channel that is activated by the Ca^{2+} influx through the Ca^{2+} ionophore. Injection of cAMP did not cause potentiation of this Cl^- current: the currents from cAMP-injected oocytes were 178 ± 27 nA (mean ± standard error, $n = 10$) and those for control oocytes were 140 ± 18 nA ($n = 10$). These data are not significantly ($P > 0.05$) from each other, thus suggesting that the Ca^{2+}-activated Cl^- current is not modulated by cAMP.

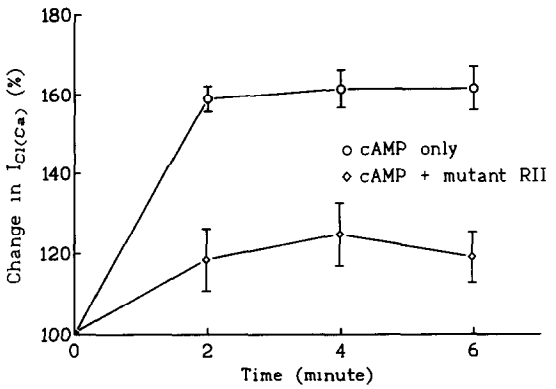

Fig. 4. Inhibition of cAMP effect by mutant PKA regulatory subunit. Oocytes were injected with cAMP (5 pmol/cell, $n = 10$, open circles) or cAMP plus the mutant RII (a mutant form of the type II regulatory subunit of PKA, 2 ng/oocyte, $n = 12$, open diamonds). $I_{Cl(Ca)}$ was recorded before and after the injection. Percent change (mean ± S.E.M.) of $I_{Cl(Ca)}$ after injection over $I_{Cl(Ca)}$ before injection was plotted against the time after injection.

3.4. *PKA is both necessary and sufficient for the cAMP effect on* $I_{Cl(Ca)}$

The results from the above experiments suggest a relationship between an elevation of intracellular cAMP and an enhancement of $I_{Cl(Ca)}$. To examine the role of the cAMP-dependent protein kinase (protein kinase A, PKA), the purified catalytic subunit of PKA was injected into oocytes, and $I_{Cl(Ca)}$ was recorded before and after the injection. As shown in Fig. 3, injection of the catalytic subunit in oocytes (70 fmol/cell, $n = 9$) increased $I_{Cl(Ca)}$. Analysis of variance showed that the $I_{Cl(Ca)}$ values were significantly different between the catalytic subunit-injected oocytes and the control ($P < 0.001$). Because the catalytic subunit is the functional moiety of PKA, these results indicate that an increase of PKA activity is sufficient to potentiate $I_{Cl(Ca)}$.

Is PKA necessary for mediating the cAMP effect on $I_{Cl(Ca)}$? To address this question, two PKA inhibitors were used. As shown in Fig. 3, when a peptide PKA inhibitor, the PKI peptide [18], was co-injected with the catalytic subunit of PKA, the enhancement of $I_{Cl(Ca)}$ by the catalytic subunit of PKA was significantly blunted ($P < 0.05$) to 56% of the enhanced level. Also, a mutant form of type II regulatory subunit of PKA (RII) was used, in which 20 residues in the autophosphorylation region were replaced by the PKI peptide sequence. This mutant RII binds strongly to the catalytic subunit of PKA to form an inactive holoenzyme which does not dissociate even in the presence of cAMP. Therefore, this mutant RII acts as a potent inhibitor of PKA activity [19]. As shown in Fig. 4, the time-dependent increase of $I_{Cl(Ca)}$ by cAMP injection (5 pmol/cell, $n = 10$) was significantly decreased when cAMP was injected together with the mutant RII (2 ng/cell, $n = 12$). Analysis of variance showed a significance level of $P < 0.001$ for all three time points. When the mutant RII was boiled before the injection, it did not have any inhibitory effect on cAMP potentiation (data not shown). These data indicate that the cAMP effect on $I_{Cl(Ca)}$ has to be mediated by PKA and can not bypass it.

3.5. *Phosphatases can suppress cAMP potentiation of* $I_{Cl(Ca)}$

To examine whether dephosphorylation could reverse the cAMP effect on $I_{Cl(Ca)}$, purified catalytic subunits of phosphatases were co-injected with cAMP into oocytes, and $I_{Cl(Ca)}$ was measured before and after the injection at different time points. As shown in Fig. 5, both type 1 and type 2A phosphatases reduced the cAMP potentiation of $I_{Cl(Ca)}$. Analysis of variance indicated that $I_{Cl(Ca)}$ was significantly different between the cAMP-injected cells and any of the three groups (two phosphatase injected and the control, $P < 0.01$ for the time points from 2 to 6 min), whereas the difference among phosphatase 1, phosphatase 2A, and the control groups was not significant ($P > 0.05$). Co-injection of boiled phosphatases with cAMP did not significantly decrease the cAMP effect on $I_{Cl(Ca)}$ (data not shown), indicating the specificity of functional phosphatases. These results suggest that the effect of cAMP on $I_{Cl(Ca)}$ is by a PKA-mediated phosphorylation and that dephosphorylation by either type of the phosphatases can reverse the effect.

4. DISCUSSION

The existence of an endogenous voltage-activated Ca^{2+} channel in *Xenopus* oocytes was first reported in the early 80's [1,17]. It is gated by membrane depolarization to a potential more positive than −20 mV, with the maximal activation at +10 to +20 mV (Fig. 1B). The

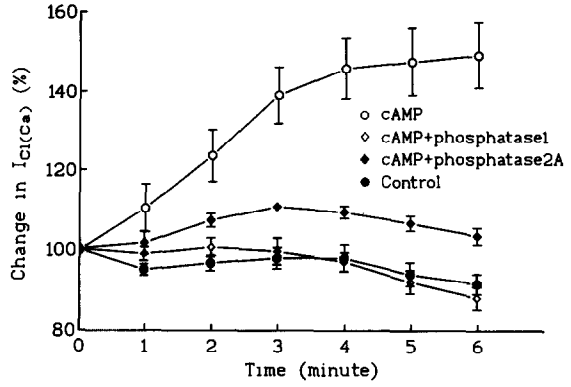

Fig. 5. Effect of phosphatases on cAMP-induced increase of $I_{Cl(Ca)}$. Oocytes were injected with cAMP alone (5 pmol/cell, $n = 5$, open circles), cAMP plus the catalytic subunit of type 1 phosphatase (2.7 fmol/cell, $n = 5$, open diamonds), cAMP plus the catalytic subunit of type 2A phosphatase (2.7 fmol/cell, $n = 5$, closed diamonds), or 10 mM Tris (pH 7.4) as control ($n = 3$, closed circles). $I_{Cl(Ca)}$ was recorded before and after the injection in each oocyte. Percent change (mean ± S.E.M.) of $I_{Cl(Ca)}$ after injection over $I_{Cl(Ca)}$ before injection was plotted against the time since injection.

opening of this channel causes a transient influx of Ca^{2+} ions and leads to the opening of a Ca^{2+}-dependent Cl^- channel [1,17]. Although the inward Ca^{2+} current per se is rather small in amplitude [2,16] (also see I_{Ba} in Fig. 2), it activates a much larger transient outward Cl^- current, $I_{Cl(Ca)}$ [1,17]. Thus, $I_{Cl(Ca)}$ carried by the Cl^- ions serves as an amplified indicator for the Ca^{2+} current through the voltage-activated Ca^{2+} channel.

It has been reported that intracellular injection of cAMP enhances $I_{Cl(Ca)}$ [2,3], suggesting a role by PKA. Here we provide the definitive evidence that PKA is the mediator of this cAMP-enhancement of $I_{Cl(Ca)}$. Both a peptide inhibitor for PKA, the PKI peptide (Fig. 3), and a mutant form of PKA type II regulatory subunit, the mutant RII (Fig. 4), inhibited the $I_{Cl(Ca)}$ enhancement, indicating a need for PKA in this process. Furthermore, purified catalytic subunit of PKA, when injected in oocytes without co-injection of cAMP, resulted in significant increase of $I_{Cl(Ca)}$ (Fig. 3). Thus, we demonstrated that PKA is both necessary and sufficient for the modulation of $I_{Cl(Ca)}$. These results also suggest that cAMP does not directly modulate $I_{Cl(Ca)}$ as has been shown for some other ion channels in the heart and olfactory epithelium [20].

Cyclic AMP injection did not result in an increase in the current through the Ca^{2+}-activated Cl^- channel when it was activated directly by Ca^{2+} influx through the Ca^{2+} ionophore, A23187. This experiment was designed to bypass any increase in the Ca^{2+} conductance caused by protein phosphorylation that might contribute to an increase in $I_{Cl(Ca)}$. These data suggest that cAMP-dependent phosphorylation does not directly modulate the Ca^{2+}-activated Cl^- channel. Thus, the oocyte Cl^- channel differs from those Cl^- channels that are modulated by cAMP-dependent protein phosphorylation [10,21]. The Cl^- channel of CFTR, for example, has been expressed in *Xenopus* oocytes and shown to be activated by cAMP-dependent phosphorylation without depolarization and Ca^{2+} influx [22].

Instead of modulating the Cl^- channel, data presented here suggest a modulation of the Ca^{2+} channel by cAMP-dependent phosphorylation. The underlying mechanism for $I_{Cl(Ca)}$ potentiation is thus mainly by an enhancement of the Ca^{2+} current through the endogenous Ca^{2+} channel (Fig. 2). Injection of purified catalytic subunits of protein phosphatases inhibited the cAMP-induced increase of $I_{Cl(Ca)}$ (Fig. 5), suggesting that PKA-mediated phosphorylation is the molecular basis for $I_{Cl(Ca)}$ modulation. The effect of phosphatase catalytic subunits does not appear to involve any endogenous inhibitory subunit for the phosphatases, since attempts to identify such inhibitory subunits in oocytes have been unsuccessful (James Maller, personal communication). Phosphorylation of Ca^{2+} channels by PKA is a well-known mechanism for modulating channel activity in the cell, often as the result of hormone and neurotransmitter activation of membrane receptors [4,5]. These receptors can modulate Ca^{2+} channel activity either by a membrane-confined, direct coupling through G proteins or by activating an effector pathway involving soluble second messengers [23]. Channel phosphorylation often leads to a larger Ca^{2+} current, due to an increased probability of channel opening, a decreased probability of channel closing, and/or a change in the number of channels from an altered rate of protein turnover [5]. The relatively quick onset of the cAMP effect in oocytes within 2 min (Fig. 4) makes the rate of channel protein turnover an unlikely cause; instead, it seems more likely the result of a functional modification of the existing channel molecules, either by a change in ion channel kinetics or by recruitment of inactive channels.

Our data do not rule out the possibility that, in addition to an increase in a voltage-gated Ca^{2+} current by cAMP, the regulation of $I_{Cl(Ca)}$ is also contributed partially through changes in Ca^{2+} buffering by cAMP-dependent phosphorylation. Phosphorylation of molecules such as phospholamban [24] involved in modulating Ca^{2+} transport into the sarcoplasmic reticulum could increase the steady-state levels of intracellular Ca^{2+}. The same amount of Ca^{2+} influx would result in higher Ca^{2+} levels in the cytosol and consequently produce an increase in the size of $I_{Cl(Ca)}$. Alternatively, phosphorylation could increase the conductance of a voltage-dependent Ca^{2+} channel in the oocyte endoplasmic reticulum, resulting in greater Ca^{2+} efflux from it upon depolarization. Such a channel has been described in the pancreatic endoplasmic reticulum [25]. Phosphorylation could also increase the Ca^{2+} sensitivity of a ryanodine receptor-like Ca^{2+}-activated Ca^{2+} channel in the oocyte endoplasmic reticulum [26]. The increase in Ca^{2+} influx from the plasma membrane Ca^{2+} channels by phosphorylation would then be multiplied by the enhanced release of Ca^{2+}. These possibilities are only likely to be answered utilizing Ca^{2+} imaging techniques, which is not the aim of this study.

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