Comparison of Activation of Non-selective Cation Channels by Protein Kinase-A in Guinea-pig and Mouse Pancreatic Acinar Cells

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Abstract: The effects of cyclic AMP-dependent protein kinase (PKA) on non-selective cation (NSC) channels in the plasma membrane of guinea-pig and mouse pancreatic acinar cells were studied by patch-clamp single-channel techniques. In both species, ATP (1.4 mM) acting on the inside of the membrane (Ca^{2+} concentration, 10^{-6} M) inhibited the opening of the NSC channel with a unit conductance of 25 pS. The addition of a catalytic unit of PKA (25 U/ml) in the presence of ATP to the inside of guinea-pig cells evoked an opening of a new channel, which is a Ca^{2+} -activated and voltage-sensitive NSC channel with a unit conductance of about 45 pS (in symmetrical monovalent cation-rich solution). On the other hand, 25 pS Ca^{2+} -activated NSC chan-

nel in mouse cells was re-opened by identical PKA application. The application of PKA-inhibitor did not activate NSC channels in either species. The addition of 10^{-6} M ACh to the bath solution containing 10^{-6} M insulin, whose addition with ACh has been reported to increase cAMP, evoked a marked and sustained increase in the opening of the guinea-pig 45 pS or the mouse 25 pS NSC channel in intact cells (cell-attached configurations). This was not seen in the absence of insulin. These results suggest that the cAMP-dependent mechanism in the pancreatic acinar cells can activate NSC channels in the presence of ATP on the inside of the cell. [Japanese Journal of Physiology, 46, 43–52, 1996]

Key words: non-selective cation channel, protein kinase-A, pancreatic acinar cell, ATP, ACh.

Ca^{2+} -dependent and voltage-insensitive cation non-selective (NSC) channels have been described in the rat, mouse [1, 2] and guinea-pig [3] pancreatic acinar (PA) cells. It is thought that the opening of the NSC channels in the basolateral membrane depolarizes PA cells, which facilitates uptake of Cl^- from the basolateral side [4].

It has been known that application of acetylcholine (ACh) or cholecystokinin (CCK) to unexcised mouse PA cells evokes the opening of NSC channels, as measured by the cell-attached patch-clamp technique [2].

While ATP exists inside the cell in physiological conditions, recent studies on excised inside-out membrane patches, which were exposed to solution containing Ca^{2+} to allow opening of the NSC channel,

have shown that ATP acting on the inside of the membrane inhibits the opening of those channels. A concentration of 1 mM ATP evokes complete channel closure in guinea-pig [3] and mouse PA cells (L. G. Squire and O. H. Petersen, personal communication).

Whether or not ACh-evoked activation of the NSC channel in mouse PA cells and the inhibition of the NSC channel due to ATP in guinea-pig and mouse PA cells are mediated by the same messenger or mechanism, though the direction of action is actually opposite in the two cases, is the subject of the present work.

Thus, the present work compares the effect of cyclic AMP-dependent protein kinase (PKA) on the closed NSC channel by previously applied ATP with

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the excised membrane patch-clamp technique in guinea-pig and mouse PA cells. It further studies the effect of ACh with insulin by the cell-attached patch-clamp technique to determine the correlation between the activation of the NSC channel and phosphorylation of the membrane protein subunit.

A brief preliminary report of some of the findings described here has already appeared [5].

METHODS

Guinea-pigs and mice were anesthetized by diethyl ether and were killed by a blow to the head. The pancreas was immediately removed. Dispersed guinea-pig acinar cells were prepared using the method described by Suzuki and Petersen [3]. Mouse acinar cells were prepared as below. We incubated small pieces of pancreas (50 mg) in physiological saline solution containing crude collagenase (Sigma type V, 1 mg/ml) for 20 min followed by a second incubation for 25 min with pure collagenase (Worthington, CLSPA, 200 U/ml). After collagenase digestion, there was a mixture of completely isolated cells and cell clusters and these were rinsed with collagenase-free physiological solution for 10–50 min before use.

Patch-clamp single-channel current recordings were obtained using the methods originally developed by Hamill *et al.* [6]. In experiments where single-channel current recording was attempted, we normally used cell clusters rather than completely isolated cells, and accessed the patch-pipette to the basolateral side (side without zymogen granules) under visual control. Membrane current records (filtered at 0.5 kHz low pass) were primarily stored on tape (Sony, KS609, Tokyo) for subsequent replay and analysis. The taped current records were digitized at 4 kHz (ELMEC, EC2390, Tokyo) and analyzed using a microcomputer (NEC, 9801 DA, Tokyo) for calculating open-state probability as described by Suzuki *et al.* [7]. The average open-state probability was determined from current records lasting 5–10 s.

The histogram of the current amplitude was obtained by measuring the amplitude of the current on the chart of the recorder (digital thermal recorder, Nihon Kohden RTA1100M, Tokyo) with a vernier caliper.

Two types of physiological saline solutions were used: a Na⁺-rich and a K⁺-rich type. The Na⁺-rich solution had the following composition (in mM): 140 NaCl, 4.7 KCl, 1.13 MgCl₂, 10 glucose, and 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.2). The K⁺-rich solution contained (in mM): 145 KCl, 10 NaCl, 1.13 MgCl₂, 10 glucose, 10

HEPES (pH 7.2). The osmolalities of the solutions were adjusted to 290±5 mOsm/kg for the Na⁺-rich and 300±5 mOsm/kg for the K⁺-rich solutions.

In the experiment where the effects of 10⁻⁷ or 10⁻⁶ M of ionized Ca²⁺ concentrations were to be tested on the membrane inside, Ca²⁺-ethylene glycol-bis-(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) buffer mixtures were used. These were prepared by titration as described by Miller and Smith [8]. The free Ca²⁺ and ATP⁴⁻ concentrations in the K⁺-rich solution containing ATP were calculated using the stability constants as described by Fabiato [9], and were adjusted to 0.1 or 1 μ M for Ca²⁺, and 10⁻⁵ M or 10⁻⁴ M for ATP⁴⁻ (total ATP concentration, 0.14 or 1.4 mM respectively) by adding Ca²⁺/EGTA mixture (total EGTA concentration, 2 mM), MgCl₂, and ATP. The free Mg²⁺ concentration was adjusted to 1 mM in all excised experiments.

The commercial catalytic unit of cAMP-dependent protein kinase (Sigma P2645) was dialyzed just before use against deionized water containing the reducing agent dithiothreitol (6 mg/ml) at 4°C for 4 h to remove buffer materials, and was then dissolved in the perfusion solution.

In some experiments, the test solution was applied by continuous flow from small-diameter (about 150 μ m) polyethylene outlet pipes. The exchange of solution from control to test was achieved manually under visual control.

Chemicals. ATP (Na₂ATP), Insulin (porcine pancreas), crude collagenase (type V), the catalytic unit of cAMP-dependent protein kinase (P2645), and a protein kinase inhibitor (P5015) were purchased from Sigma, U.S.A. Pure collagenase was purchased from Worthington, U.S.A. Tetraethylammonium (bromide) was purchased from Wako Pure Chemical Industries, Japan. All other chemicals used were of reagent grade.

RESULTS

Effect of protein kinase A on NSC channels in guinea-pig

Acinar cells in the guinea-pig possess Ca²⁺-activated NSC channels with a unit conductance of about 25 pS, and two kinds of Ca²⁺- and voltage-activated K⁺-selective channels with unit conductances of about 30 pS and 200 pS, respectively [3]. Figure 1 shows single-channel current recording of the NSC channel from an excised inside-out membrane patch exposed to K⁺-rich solution on both sides of the membrane. In the experiment where the free Ca²⁺ concentration in the bath solution (inside of mem-

Fig. 1. Single-channel currents recorded from an excised inside-out membrane patch exposed to K^+ -rich solution on both sides of the membrane. The pipette solution contained $1.2 \text{ mM } Ca^{2+}$, whereas the bath fluid contained Ca^{2+} -EGTA mixture giving a free $[Ca^{2+}]$ of 10^{-6} M . The membrane potential was clamped at the voltage values indicated. All traces were obtained from the same

membrane patch in the absence of ATP (left column), and 1 min after the start of exposure to the solution containing $10^{-5} \text{ M } ATP^{4-}$ (middle column), and 5 min after the start of exposure to 25 U/ml PKA and $10^{-5} \text{ M } ATP^{4-}$ (right column). ---, current level when all channels are closed. Upward deflections represent outward current.

brane) ($[Ca^{2+}]_i$) was kept constant at 10^{-6} M (Fig. 1, left column), $10^{-5} \text{ M } ATP^{4-}$ (total ATP, 0.14 mM) abolished the opening of the NSC channels (Fig. 1, middle column). We examined whether PKA can activate the NSC channel after inhibition of the NSC channel just shown. One minute after the addition of 25 U/ml catalytic subunit of cyclic AMP-dependent protein kinase (PKA) to the inside of the membrane of the same patch, openings of new channel were seen. Five minutes after the application, the openings of the channel were sustained (Fig. 1, right column). The openings due to PKA continued throughout the observation (for 30 min). A similar opening of the channel by PKA was seen in five out of eight patches in which the ex-

periment was attempted. The latencies of the opening of the channels in five experiments were between 1 min and 5 min.

Figure 2A shows amplitude histograms of single-channel currents at $10^{-6} \text{ M } Ca^{2+}$ (without ATP and PKA) (left panel) and after PKA application (right panel) at a membrane potential of 40 mV . The current level at which the peak occurred without ATP and PKA was between 1.0 pA and 1.1 pA at a membrane potential of 40 mV , which indicates a unit conductance of 25 pS . The current level at which the peak occurred in PKA application was between 1.7 pA and 1.8 pA , which indicates a unit conductance of 45 pS .

In the experiments on PKA application, the ampli-

Fig. 2. A: Amplitude histograms of single-channel current at a membrane potential of 40 mV. All events were obtained from the results of single-channel currents in excised inside-out configurations including Fig. 1. The left and right panels show the histograms obtained from the currents without ATP and PKA (10^{-6} M Ca^{2+}) and after PKA application, respectively. **B: Single-channel current-voltage relationships obtained from 3 different ionic condi-**

tions on excised inside-out patches. K/K, K-rich solution on both sides. Na/K, Na^+ -rich solution in pipette, K^+ -rich solution in the bath. Cl/SO₄, KCl solution in the pipette, K_2SO_4 solution in the bath with the same K^+ concentration (145 mM) on both sides (sulfate solution balanced with sucrose). Same symbols represent the results obtained from the same membrane patch.

tude of the single-channel currents varied linearly with voltage. The relationships of current-voltage for the new channel in the experiment using K^+ -rich solution on both sides of the membrane were slightly outwardly rectifying. The mean unit conductances were 44.7 ± 4 for outward and 28.0 ± 3 for inward currents (mean $\pm$ SE, $n=5$) (Fig. 2B, left panel). Similar results were obtained using Na^+ -rich solution in a pipette and K^+ -rich solution in a bath (Na/K) ($n=3$), and using K^+ -sulfate solution in the bath and KCl in the pipette (same K^+ concentration on both sides) (Cl/ SO_4) ($n=3$) (Fig. 2B, right panel). The mean unit conductances for two ionic conditions, Na/K and Cl/ SO_4 in Fig. 2B (right panel), were 43.5 ± 3.4 for outward and 28.5 ± 1.7 for inward currents (mean $\pm$ SE, $n=6$).

On two conditions of Ca^{2+} -gradient between outside and inside the membrane, 1.2 mM/1 μ M (out/in) and 0.1 μ M/1 μ M (out/in), there was no change in the reversal potential (not shown). Results from these experiments show that a new channel after the application of PKA was permeable to Na^+ and K^+ and impermeable to Cl^- , and its Ca^{2+} permeability was either very low or absent. Thus, the new channel possesses features of non-selective monovalent cation channels.

At $[Ca^{2+}]_i = 10^{-6}$ M, openings of the NSC channel due to PKA application were recorded at a membrane potential of -40 mV and positive membrane potentials were needed to observe long channel openings. When $[Ca^{2+}]_i = 10^{-7}$ M, more positive membrane potentials were needed to activate the channel (not shown).

To check whether PKA can evoke openings of the new NSC channel in the presence of higher concentrations of ATP on the inside face of an inside-out membrane patch, a bath was filled with K^+ -rich solution containing 10^{-4} M ATP^{4-} (total ATP, 1.4 mM) ($[Ca^{2+}]_i = 10^{-6}$ M) and a pipette contained K^+ -rich solution with 0.5 mM EGTA. Three minutes after PKA application, sustained openings of the NSC channel were observed at positive membrane potentials (not shown). This experiment was repeated 3 times with similar results.

The activation of new NSC channels due to PKA was not seen in the absence of ATP; thus, PKA alone was ineffective. The application of PKA in the presence of 100 μ M of a PKA inhibitor (from rabbit muscle) did not evoke opening of the channel ($n=4$) (not shown).

Although it seems that the cAMP-dependent mechanism is necessary for the opening of the 45 pS new NSC channel, it is known that stimulation of PA cells with acetylcholine (ACh) or pentagastrin evokes only

an increase in $[Ca^{2+}]_i$ but not in cyclic AMP. On the other hand, stimulation with ACh in the presence of a low insulin concentration evokes an increase in cyclic AMP accompanied with an increase in $[Ca^{2+}]_i$ [10]. Thus, we tested whether ACh stimulation in the presence of insulin can evoke NSC channel opening in intact cells (cell-attached configurations). A pipette was filled with K^+ -rich solution containing 10 mM tetraethylammonium (TEA) which has been reported to inhibit the opening of K^+ channels [3]. A bath was filled with Na^+ -rich solution. Three minutes after the addition of 10^{-6} M ACh acting outside the electrically isolated membrane area from which single-channel current recording is carried out, there was no increase in the frequency of channel opening (Fig. 3A, middle column). After the addition of insulin to the bath solution, 1 min after the identical application of ACh, there was an increase in the frequency of channel opening. This was sustained at 3 min after the application of ACh (Fig. 3A, right column). The openings were seen throughout the observation period (for 15 min). A similar sustained opening of the channel was seen in four out of seven patches in which the experiment was attempted. The latencies of the channel opening were between 1 and 4 min. After the bath solution was washed away, the readmission of ACh with insulin did not evoke a sustained opening of the channel. Open-state probabilities (p) were increased by depolarizing the membrane (toward more negative pipette potential, V_p) (Fig. 3B). The application of insulin alone was ineffective.

Figure 3C shows the current-voltage relationships obtained from experiments on cell-attached configurations in three different ionic conditions in pipette (including experiment shown in Fig. 3A). The evoked currents were obtained by ACh stimulation in the presence of insulin in the bath solution. In three different pipette solutions, K^+ -rich, Na^+ -rich, and Cl^- -free solutions, all points fell on the same line, the slope of which corresponds to single-channel conductances of 44 pS for outward current and 31 pS for inward current. The results suggest that these channels are permeable to Na^+ and K^+ , and impermeable to Cl^- .

Effects of PKA on NSC channel in mouse cells

To test whether PKA can activate the mouse NSC channel after inhibition of the channel opening due to ATP, patches of plasma membrane were excised in the inside-out configuration. The bath was filled with K^+ -rich solution containing Ca^{2+} /EGTA buffer mixtures adjusted to give a 10^{-6} of $[Ca^{2+}]_i$, whereas the pipette was filled with Na^+ -rich solution containing 1.2 mM

Fig. 3. A: Single-channel currents recorded from intact acinar cells (cell-attached configurations). Pipette potential (V_p) was clamped at the voltage indicated to the left of the traces. The pipette was filled with K^+ -rich solution containing 10 mM tetraethylammonium. The bath was filled with extracellular Na^+ -rich solution. All traces were obtained from the same membrane patch before ACh stimulation (control), 3 min after the addition of $10^{-6} M$ ACh to the bath solution (ACh), and 3 min after the application of ACh in the presence of $10^{-6} M$ insulin in the bath solution

(insulin+ACh). **B: Open-state probability as a function of pipette potential in the experiment shown in Fig. 3A. C: Relationships between single-channel current and pipette potential after ACh stimulation in the presence of insulin in the three separate experiments (including A) using intact cells.** K, Na, and SO_4 represent K^+ -rich solution, Na^+ -rich solution, and Cl^- -free solutions (K_2SO_4) in the pipette, respectively. The same symbol represents the results obtained from the same patch.

Fig. 4. Single-channel current recording from excised inside-out membrane patch at membrane potential (MP) of -20 mV. The pipette was filled with Na^+ -rich solution containing 1.2 mM Ca^{2+} . The bath was filled with K^+ -rich solution containing Ca^{2+} -EGTA buffer adjusted to give a

10^{-6} M of $[\text{Ca}^{2+}]_i$. All current recordings were obtained from the same membrane patch with no ATP-containing solution (10^{-6} M Ca^{2+}), and ATP-containing (10^{-4} M ATP^{4-}) solution (ATP-containing solution) in the bath. Arrow indicates the addition of 25 U/ml PKA to the bath solution.

Ca^{2+} . The NSC channels with a unit conductance of about 25 pS were observed. The bath solution containing 10^{-4} M ATP^{4-} (total ATP, 1.4 mM) blocked opening of the NSC channels completely. Thirty seconds after the addition of 25 U/ml PKA to the inside of the membrane, the channel was again opened (Fig. 4). This channel was equally permeable to Na^+ and K^+ and impermeable to Cl^- . The channel possessed voltage-insensitive properties for channel opening, and had a unit conductance of 25 pS without rectification (not shown). A similar activation of the 25 pS channel was seen in five out of nine patches in which the experiment was attempted. The activation of the NSC channels took place between 30 s and 3 min. The activation of the NSC channel was not seen in the absence of ATP. PKA alone was ineffective ($n=3$). The application of PKA in the presence of 100 μM of a PKA inhibitor did not activate the NSC channel (Fig. 5) ($n=4$).

We tested intact cells to see whether there is a difference in NSC channel openings between in the presence and absence of insulin in addition to ACh in the bath solution. The pipette was filled with K^+ -rich solution, whereas the bath was filled with Na^+ -rich solution containing 1.2 mM Ca^{2+} . Thirty seconds after the addition of 10^{-6} M ACh to the bath solution containing 10^{-6} insulin, there was an increase in the frequency of channel opening. This was sustained at 3 min after the application of ACh (Fig. 6A, right column). A similar sustained opening of the channel was seen in three out of six patches in which the experiment was attempted. The latencies of the channel opening were between 30 s and 3 min. In the absence of insulin, there was not a sustained increase. In both experiments in the presence and absence of insulin, the number of available channels was mostly one. The

Fig. 5. Single-channel currents were recorded from excised inside-out membrane patches at a membrane potential (MP) of -20 mV. The pipette was filled with Na^+ -rich solution containing 1.2 mM Ca^{2+} . The bath was filled with K^+ -rich solution containing Ca^{2+} -EGTA mixtures giving a 10^{-6} M of $[\text{Ca}^{2+}]_i$. All current recordings were obtained from the same membrane patch. The top and middle traces were obtained in the absence of ATP (10^{-6} M Ca^{2+}), and 1 min after the addition of 10^{-4} M ATP^{4-} to the bath (ATP). The bottom trace was obtained 3 min after the start of exposure to 25 U/ml PKA and 100 μM PKA-inhibitor with ATP (PKA+inhibitor).

application of insulin alone was ineffective.

Figure 6B shows the relationships of pipette voltage-current on cell-attached configurations in three different ionic conditions in a pipette. The currents were obtained by ACh stimulation in the presence of insulin in the bath solution. In three different pipette solutions, K^+ -rich, Na^+ -rich, and Cl^- -free solutions, all points fell on the same line, the slope of which corresponds to a conductance of 25 pS. These results suggest that these channels are permeable to Na^+ and K^+ , and impermeable to Cl^- .

A

B

Fig. 6. A: Single-channel currents recorded from intact acinar cells (cell-attached configurations). Pipette potential (V_p) was clamped at the voltages indicated to the left of the traces. The pipette was filled with K^+ -rich solution, whereas the bath was filled with extracellular Na^+ -rich solution. All traces were obtained from the same membrane patch before ACh stimulation (control), 3 min after the addition of $10^{-6}M$ ACh to the bath solution (ACh), and 3 min after the application of ACh in the presence of $10^{-6}M$ insulin in the bath solution (insulin+ACh). **B: Relationships between single-channel current and pipette potential after ACh stimulation in the presence of insulin in three separate experiments (including A) on intact cells (cell-attached configurations).** K, Na, and SO_4 represent K^+ -rich solution, Na^+ -rich solution, and Cl^- -free solution (K_2SO_4) in the pipette, respectively. The same symbol represents the results obtained from the same patch.

DISCUSSION

Guinea-pig pancreatic acinar cells. Although it has been reported that K^+ -selective channels are inhibited by intracellular high concentration of ATP (more than 1 mM) [11, 12], the present results showed that 1.4 mM ATP did not block the opening of the new NSC channel due to PKA application; on the contrary, ATP was necessary for activation of the new

channel in excised membrane fragments. Thus, our results obtained with excised membrane fragments provide evidence for the activation of NSC channels by cAMP-dependent phosphorylation. We cannot determine whether the NSC channel protein itself is phosphorylated or whether a separate regulatory subunit is phosphorylated. The activation on excised patches indicates that the phosphorylated protein remains closely associated with the channel, even when the

patch membrane is removed from the cell.

In the instance where channel activation occurs, the NSC channel with a unit conductance of 25 pS (previously existing NSC channel) was not activated by PKA, but another NSC channel with a unit conductance of about 45 pS (new NSC channel) was now opened. We cannot rule out the possibility that the unit conductance of the previous NSC channel and the voltage-insensitivity of the channel were changed by PKA application.

The sub-peak (0.3–0.4 pA) of the histogram of current amplitude for new 45 pS NSC channel (Fig. 2A, right panel) did not coincide with the main peak for previously existing 25 pS NSC channels. The sub-peak may indicate the current amplitude of the sub-state of full-opening for the 45 pS channel.

In the experiments under inhibition of the K^+ channel, ACh stimulation could evoke an opening of the new NSC channel in intact cells (Fig. 3). Intracellular microelectrode recordings have shown that stimulation with secretagogues typically evokes a biphasic membrane potential change, namely an initial depolarization associated with little change in membrane resistance followed by a prolonged hyperpolarization with a reduction in membrane resistance [13]. It is possible that the initial depolarization seen in the microelectrode experiments is due to the opening of the 45 pS NSC channel.

Mouse pancreatic acinar cells. It has been reported that the 25 pS NSC channel in the mouse cells can be opened even by the application of ACh alone (without insulin) in cell-attached configurations [2]. Our results showed that ACh application evoked an opening of the mouse 25 pS NSC channel with a latency shorter than that of the 45 pS channel in guinea-pig cells. The delayed opening of the guinea-pig NSC channel suggests that the time required for the opening of the 45 pS channel may be longer due to the participation of a cAMP-dependent mechanism.

The results obtained from PKA and PKA-inhibitor applications suggest that cAMP-dependent phosphorylation can also activate the NSC channel in mouse PA cells. PKA activated the 25 pS NSC channel in mouse cells but not in guinea-pig cells. The reason for these differences is not clear, but the kinetics of the channel protein itself or a separate regulatory subunit may be different.

In the present preparation, the sensitivity of mouse NSC channel opening to internal Ca^{2+} was low (Figs. 4 and 5) compared with that of the NSC channel (25 pS conductance) in guinea-pig PA cells (Fig. 1, left column). Similar results for Ca^{2+} -sensitivity have been previously shown in mouse PA cells [14] and

lacrimal acinar cells [15] in excised patches. Responses to ACh at concentrations between 10^{-7} and 5×10^{-7} M have been reported in intact mouse PA cells using a calcium-sensitive fluorescent probe [16]. In intact cells, ACh stimulation accompanied with insulin could evoke more frequent opening of the NSC channel than that of ACh stimulation without insulin. These results suggest that involvement of the cAMP-pathway can further activate the NSC channel, and that the NSC channel in intact cells is more sensitive to Ca^{2+} than hitherto shown in excised patches.

The present study results suggest that the presence of physiological ATP is necessary for cAMP-dependent phosphorylation in intact cells, though ATP inhibits the opening of the NSC channel in excised membrane fragments.

There is evidence that cAMP-dependent phosphorylation increases the Ca^{2+} -sensitivity of K^+ channels [17–19, 20]. cAMP-dependent phosphorylation is also involved in Cl^- channel activation [21–23]. Furthermore, non-selective cation channel in fetal distal lung epithelium is activated by the participation of the cAMP-pathway due to catecholamine application [24]. The data obtained from different channels together suggest that phosphorylation is involved in each individual channel activation step. cAMP-dependent phosphorylation might be essential for maintenance of normal non-selective cation channel activity.

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