

A hyperpolarization- and acid-activated nonselective cation current in *Xenopus* oocytes

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Kuruma, Akinori, Yoshiyuki Hirayama, and H. Criss Hartzell. A hyperpolarization- and acid-activated nonselective cation current in *Xenopus* oocytes. *Am J Physiol Cell Physiol* 279: C1401–C1413, 2000.—Heterologous expression of a variety of membrane proteins in *Xenopus* oocytes sometimes results in the appearance of a hyperpolarization-activated inward current. The nature of this current remains incompletely understood. Some investigators have suggested that this current is a Cl current, whereas others have identified it as a nonselective cation current. The purpose of this investigation was to characterize this current in more detail. The hyperpolarization-activated inward current (I_{IN}) present in native oocytes was composed of a current carried at least partly by Ca and Mg under physiological ionic conditions plus a Ca-activated Cl current. The Ca-activated Cl current was blocked by chelation of cytosolic Ca with 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid. When Cl currents were blocked, the cation current could be carried by Ca, Mg, or Co, but not appreciably by Ba, Mn, or Cd. I_{IN} was stimulated by intracellular acidification. The properties of I_{IN} were quite different from those of the store-operated Ca current. Heterologous expression of transient receptor potential-like gene product (TRPL), one of the members of the transient receptor potential family of putative store-operated Ca channels, apparently resulted in alteration of the voltage sensitivity of the endogenous I_{IN} .

chloride current; magnesium; divalent cation; D-*myo*-inositol 1,4,5-trisphosphate; calcium chelator

XENOPUS LAEVIS OOCYTES are one of the most widely used heterologous expression systems for studying the properties of cloned ion channels (3–5, 7, 16, 22, 32, 35). Injection of oocytes with cRNA into the cytoplasm or cDNA into the nucleus results in the expression of ion channels in the plasma membrane between several hours to several days later. These cloned channels can then be studied by two-microelectrode voltage clamp or patch-clamp analysis.

Experiments on cloned channels expressed in heterologous cell types can be complicated by the existence of endogenous channels. Endogenous channels may either be upregulated in response to expression of the exogenous protein or may form heteromultimers with the exogenous channel. For example, the expression of a G protein-gated inwardly rectifying K (GIRK1) cur-

rent on injection of GIRK1 cRNA into oocytes depends on the presence of an endogenous inwardly rectifying K channel called XIR (10).

A number of investigators have reported that expression of exogenous ion channels or other membrane proteins in *Xenopus* oocytes results in the appearance of a hyperpolarization-activated current (2, 15, 34, 36). This hyperpolarization-activated current is enigmatic. Although a very similar current is present in native oocytes (27), the exact properties of this current have been reported to depend on the properties of the expressed ion channel (15). Furthermore, although most investigators conclude that this current is a Cl current (2, 15, 27, 34), Tzounopoulos et al. (36) show that in the absence of extracellular Ca, this channel appears to behave as a nonselective cation channel.

The presence of this endogenous hyperpolarization-activated current may affect the interpretation of expression studies. For example, recently, Schmieder et al. (33) reported that the currents induced by expression of the *Xenopus* homologue of ClC-5 (xClC-5) in *Xenopus* oocytes depended significantly on the vector used. If the cRNA was flanked by *Xenopus* β -globin 5'- and 3'-untranslated regions, the anion selectivity and sensitivity to Cl channel blockers was different than when the cRNA contained the native xClC-5 untranslated regions. The authors suggest that the current induced by the β -globin-flanked cRNA was genuine xClC-5 current, whereas the current induced by the native cRNA was due to upregulation of endogenous Cl currents.

To interpret expression studies with cloned ion channels in *Xenopus* oocytes objectively, particularly Cl channels, it is necessary to have a more thorough understanding of the endogenous currents in the oocyte. In this paper, we show that the hyperpolarization-activated current is primarily a cation channel that is permeable to Ca ions. The entry of Ca ions through this channel secondarily activates Ca-activated Cl currents that we and others have studied in detail (9, 17, 18, 20, 21). This channel has a number of interesting features: the channel is activated by cytosolic acidification and is highly permeable to Mg ions.

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METHODS

Electrophysiological Methods

Xenopus oocytes were voltage clamped with two microelectrodes using an Axon Instruments GeneClamp 500. Electrodes were filled with 3 M KCl (or 4 M potassium acetate for experiments on the store-operated Ca current, I_{SOC}) and had resistances of 0.5–2 M Ω . The bath was grounded via a 3 M KCl agar bridge (4 M potassium acetate for I_{SOC} experiments) connected to a chlorided Ag wire. Typically, the membrane was held at -35 mV, and voltage steps were applied as described in the text. Stimulation and data acquisition were controlled by pCLAMP 6.01 (Axon Instruments) via a Digidata 1200 A-D/D-A converter (Axon Instruments) and a Gateway P5–90 computer (Intel Pentium, 90 MHz). During recording, the oocyte was superfused with normal Ringer solution. The bath chamber volume was ~ 300 μ l and was superfused at a rate of 2 ml/min unless the composition of the solution was being changed, in which case superfusion rates as high as 15 ml/min were used. When the composition of the bath was to be changed, the superfusion rate of the control solution was increased for several minutes before changing to the new solution at the same flow rate. Experiments were performed at room temperature (22–26°C). For experiments on I_{SOC} , oocytes were soaked overnight in Cl-free storage solution, and the experiments were performed in Cl-free solution. Ca stores were depleted by a supramaximal injection of D-myo-inositol 1,4,5-trisphosphate (IP₃). The data in the figures were not corrected for liquid junction potentials, but the data in the text were corrected for liquid junction potentials as indicated. Junction potentials relative to normal Ringer solution measured as described by Neher (25) were: 0 mV for 90 mM Cl Ringer solution, +5 mV for 23 mM Cl Ringer solution, -4 mV for N-methyl-D-glucamine (NMDG)-Cl Ringer solution, +8 mV for 0-Cl Ringer solution, and +4 mV for NMDG-aspartate Ringer solution. For tail current analysis, data were recorded at the maximum gain of the amplifier ($\times 10,000$) with the minimum phase lag (< 200 μ s) in the feedback circuit and minimum electrode resistance (0.5–1 M Ω), to maximize the frequency of the response of the voltage clamp. Still, capacitive transients usually prohibited the measurement of tail currents accurately before 8 ms.

Microinjection

Oocytes were sometimes injected with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), EGTA, or IP₃ using a Drummond Nanoject automatic oocyte injector. The injection pipette was pulled from glass capillary tubing in a manner similar to the recording electrodes, and was then broken so that it had a beveled tip with an inside diameter of < 20 μ m. We usually injected 23 nl of a 50 mM solution of K₄BAPTA or 23 nl of 100 mM Na₂K₂EGTA in water to give a final calculated concentration in the oocyte of 1–2 mM. The injection pipettes were usually left impaled in the oocyte for the duration of the experiment. When IP₃ was injected, typically, 4.6 nl of 10 mM IP₃ in H₂O was injected to give a calculated oocyte concentration of ~ 50 μ M.

Solutions

Normal Ringer consisted of 123 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1.8 mM MgCl₂, and 10 mM HEPES (pH 7.4). Ringer (90 mM Cl) consisted of 82 mM NaCl, 2 mM MgCl₂, 2 mM CaCl₂, and 10 mM NMDG-HEPES (pH 7.4). Ringer (23 mM Cl) consisted of 15 mM NaCl, 63 mM sodium aspartate,

2 mM MgCl₂, 2 mM CaCl₂, and 10 mM HEPES (pH 7.4). NMDG-Ringer consisted of 90 mM NMDG-Cl, 5 mM CaCl₂, and 5 mM NMDG-HEPES (pH 7.4). NMDG-aspartate Ringer consisted of 90 mM NMDG-aspartate, 5 mM Ca(OH)₂, and 5 mM NMDG-HEPES (pH 7.4). Cl-free storage solution was 108 mM sodium aspartate, 1.6 mM potassium aspartate, 10 mM sodium HEPES, 2 mM Ca(OH)₂, and 2 mM MgSO₄ (pH 7.4).

Harvesting Oocytes

All procedures were performed in accordance with the Institutional Animal Care and Use Committee and NIH guidelines. Stage V-VI oocytes were harvested from adult *X. laevis* females (*Xenopus* I) as described by Dascal (4). *Xenopus* were anesthetized by immersion in tricaine (1.5 g/l). Ovarian follicles were removed, cut into small pieces, and digested in 0-Ca Ringer that contained 2 mg/ml collagenase type IA (Sigma) for 2 h at room temperature. The oocytes were extensively rinsed and placed in L15 medium (GIBCO) and stored at 18°C. Oocytes were usually used between 1 and 6 days after isolation.

Synthesis of cRNA

Transient receptor potential-like gene product (TRPL) in PBS was linearized with *Not* I restriction endonuclease. Capped cRNA was synthesized in vitro using the Ambion mMessage mMachine in vitro transcription kit and T7 RNA polymerase. cRNA was analyzed by denaturing formaldehyde gel electrophoresis and found at the expected size (~ 3.2 kb).

Display and Analysis of Data

For display of the figures, current transients during voltage steps were often blanked for 5 ms. Data points are the mean, and error bars are \pm SE. Each current-voltage (I - V) and activation curve is the average of 3–6 different oocytes. For I - V relationships, the raw data were averaged. For the activation curves, the data were normalized such that the current at a particular voltage was set as 1.0. Mathematical fits were performed using an iterative Levenberg-Marquardt algorithm. Unless otherwise noted, the interval between stimuli was 10–20 s. In tail current analysis, one would like to be able to measure the current immediately after the voltage step; however, because of the capacitive transient, we were not able to measure the instantaneous current sooner than 5–8 ms after the step.

RESULTS

An Endogenous Hyperpolarization-Activated Current in Xenopus Oocytes

Hyperpolarization to -140 mV from a holding potential of -35 mV elicited only a small time-independent current. However, hyperpolarization to potentials negative to -150 mV usually elicited a time-dependent inward current. This hyperpolarization-activated inward current was termed I_{IN} (Fig. 1A). On repolarization to $+20$ mV, a time-dependent outward current (I_{OUT}) developed that decayed back to baseline over the next few seconds. The amplitude of I_{IN} was variable, as reported by others (34, 36). In our experience, however, I_{IN} at -200 mV was seldom > 1 μ A (> 100 oocytes from ~ 10 donors). To determine the activation curve for I_{IN} ,

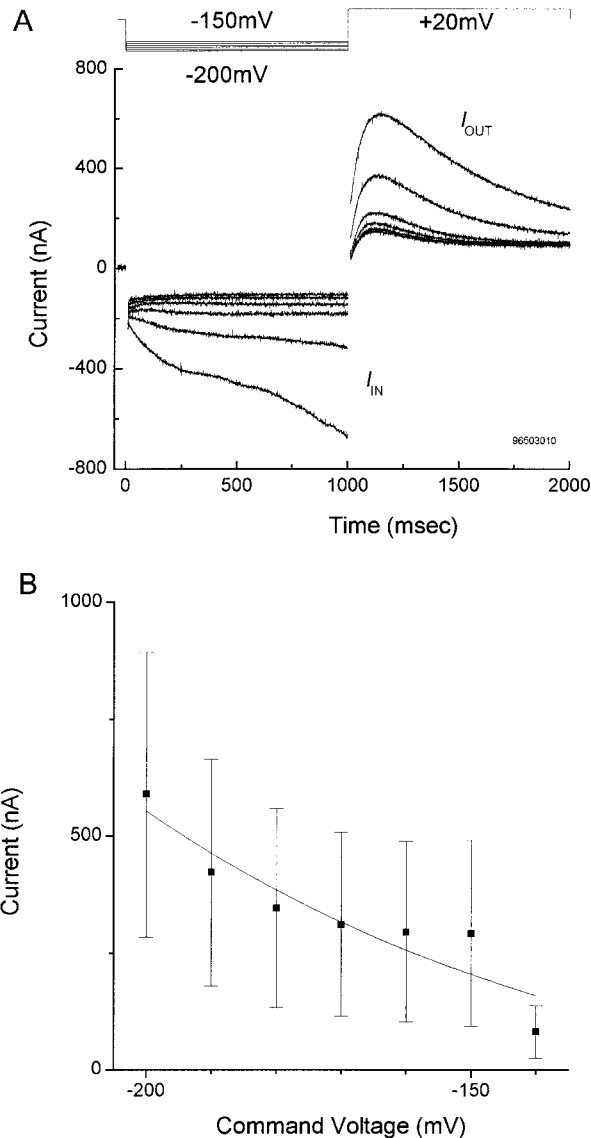


Fig. 1. An endogenous hyperpolarization-activated inward current is present in *Xenopus* oocytes. *A*: current traces. *Xenopus* oocytes were bathed in normal Ringer solution and voltage clamped with 2 microelectrodes from a holding potential of -35 mV. The voltage-clamp episode consisted of a 1-s step to potentials between -200 mV and -150 mV, followed by a 1-s step to $+20$ mV. Episodes were repeated every 20 s. The largest inward and outward currents correspond to the -200 -mV pulse. *B*: average tail current amplitudes. Tail currents were measured 8 ms after the onset of the $+20$ mV-pulse and plotted against the potential of the preceding hyperpolarizing pulse. The solid line is the best fit of a single exponential to the data. I_{IN} , hyperpolarization-activated inward current; I_{OUT} , time-dependent outward current.

we measured the instantaneous current (at 8 ms after the onset of the voltage pulse, before the I_{OUT} had developed significantly) after repolarizing to $+20$ mV from different test potentials between -200 mV and 0 mV. The instantaneous current was plotted as a function of the prepulse potential. Figure 1*B* shows the average tail current amplitudes after the time-independent component had been subtracted. On average, the tail current at $+20$ mV from a -160 -mV step was

$0.24 \pm 0.2 \mu\text{A}$ ($n = 24$). The inward current showed no signs of saturation with time or voltage.

I_{IN} Is a Mixture of a Cation Current and a Ca-Activated Cl Current

I_{IN} is only partially blocked by BAPTA injection. *Xenopus* oocytes have a very high concentration of Ca-activated Cl channels (9). To test whether I_{IN} involved a Ca-activated Cl current, we injected the oocyte with BAPTA to a final concentration of ~ 1 mM. Although this concentration of BAPTA completely blocks the Ca-activated Cl currents (9), it only partially blocked I_{IN} , whereas I_{OUT} was almost completely blocked (Fig. 2, *A* and *B*). These data led us to hypoth-

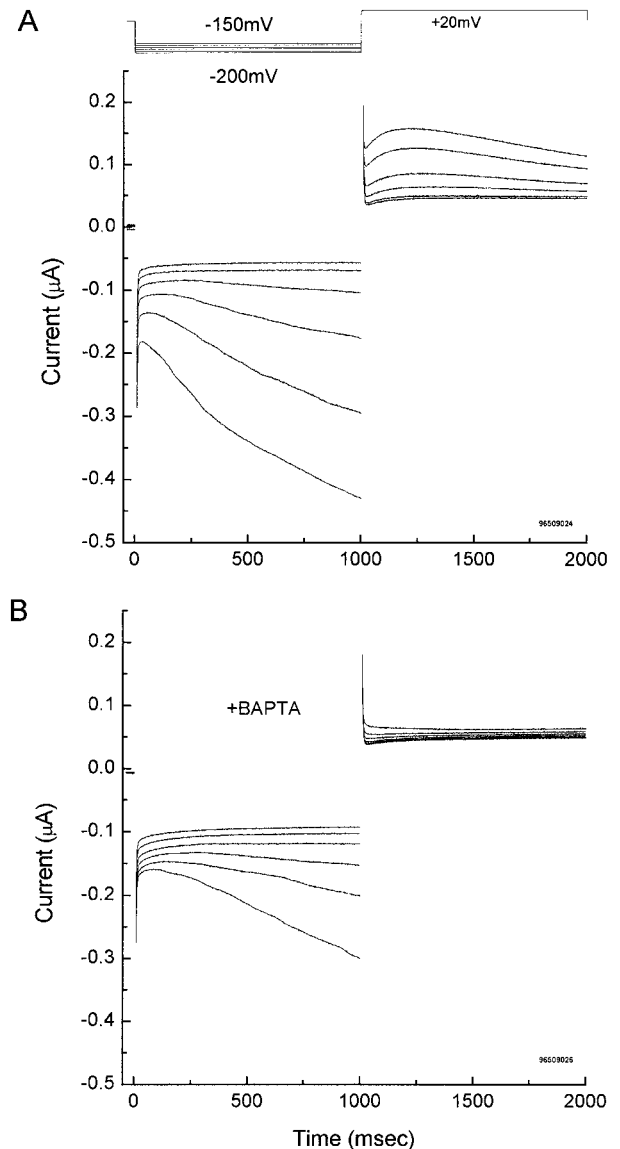


Fig. 2. Effects of intracellular 1,2-bis(2-aminophenoxy)ethane- N,N,N',N' -tetraacetic acid (BAPTA) on hyperpolarization-activated current. The experimental setup was the same as in Fig. 1. Oocytes were injected with 23 nl of a 50 mM solution of K_4 BAPTA to give a final estimated intracellular concentration of ~ 1 mM. *A*: current traces before BAPTA injection. *B*: current traces after BAPTA injection.

esize that I_{IN} was composed of two components: 1) a Ca-permeable cation current, and 2) a Ca-activated Cl current that was activated as a consequence of the Ca influx through the cation conductance.

We show (see Fig. 5) that I_{OUT} is a Ca-activated Cl current. The fact that this current was activated after I_{IN} supports the suggestion that Ca influx occurred during I_{IN} . The idea that the inward current was carried partly by Ca was supported by the observation that the BAPTA-insensitive inward current was blocked by divalent and trivalent cations including Cd, Gd, and La. I_{IN} in the presence of Ca ions was also blocked 70–80% by 10 μ M Gd ($n = 6$) and was blocked by La with an IC_{50} of $\sim 10 \mu$ M ($n = 5$; data not shown).

The reversal potential of I_{IN} suggests multiple ionic permeabilities. To determine the current-carrying ionic species more rigorously, we measured the sensitivity of the reversal potential to extracellular [Cl] ($[Cl]_o$). The instantaneous I - V relationship for the inward current was determined by tail current analysis (Fig. 3A). The membrane was hyperpolarized to -200 mV for 1 s and then repolarized to different test potentials. The current 8 ms after stepping to the test potential was plotted as a function of the test potential with 90 mM $[Cl]_o$ (Fig. 3A) or 23 mM $[Cl]_o$ (Fig. 3B). The I - V relationships are plotted in Fig. 3C. After correcting for liquid junction potentials (25), the reversal potential of the current shifted 15 mV (from -28 mV with 90 mM

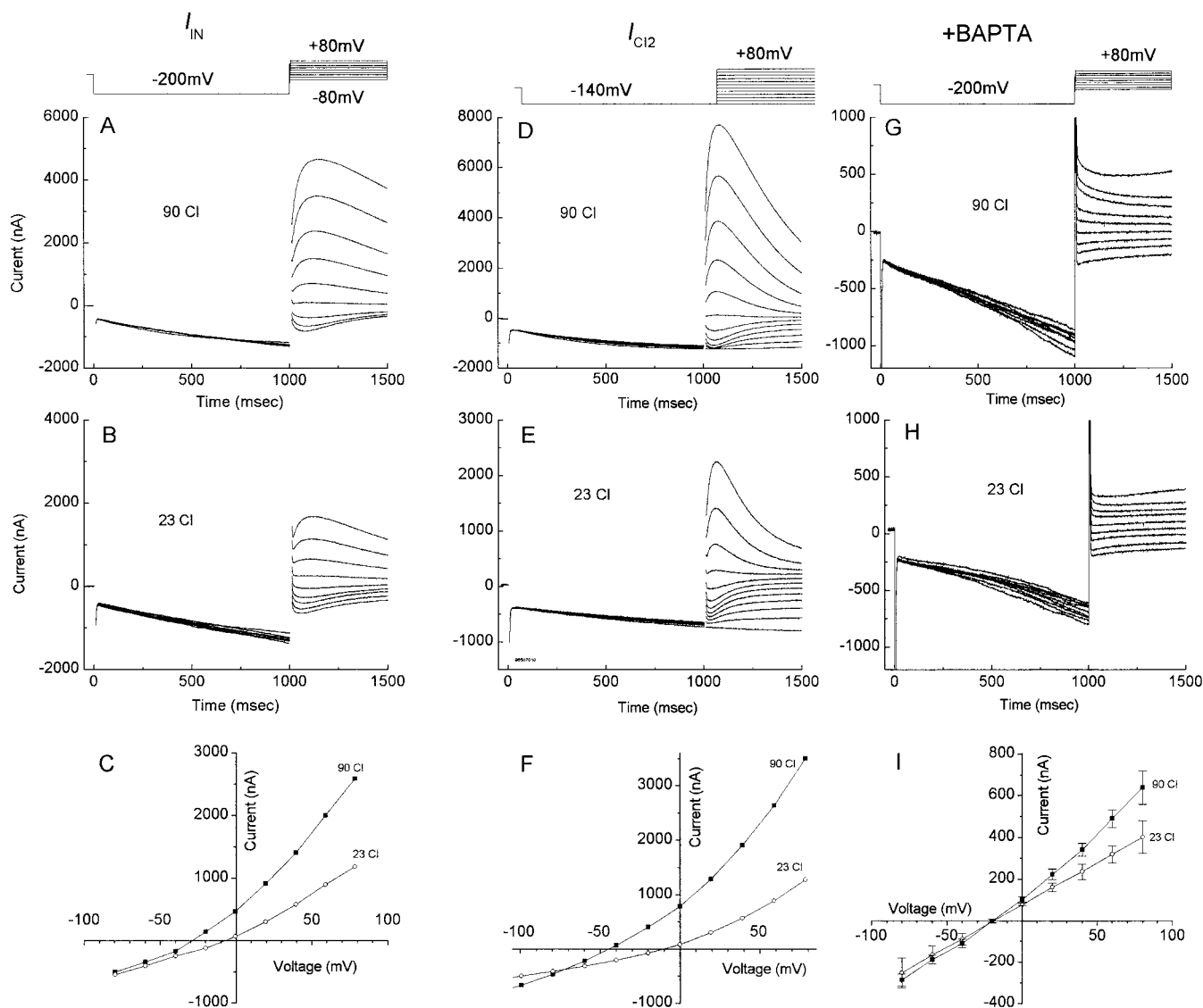


Fig. 3. Effect of changing extracellular [Cl] on hyperpolarization-activated current (I_{IN} ; A–C), the Ca-activated Cl current I_{Cl2} (D–F), and I_{IN} in BAPTA-injected oocytes ($n = 3$; G–I). Oocytes were bathed in 90 mM Cl-containing solution. A, D, and G: current traces of oocytes bathed in 90 mM Cl-containing solution. B, E, and H: current traces of currents in 23 mM Cl-containing solution. C, F, and I: instantaneous current-voltage (I - V) relationships measured by amplitude of the tail current at $+20$ mV 8 ms after the end of the hyperpolarizing pulse for the currents in 90 mM Cl (■) and 23 mM Cl (○). I_{Cl2} (D–F) was measured in an oocyte that had been injected ~ 10 min earlier with 23 nl of 1 mM D-*myo*-inositol 1,4,5-trisphosphate (IP_3). In G–I, the oocytes were injected with 23 nl of 50 mM BAPTA 10 min before the records were taken.

Cl_o to -13 mV with 23 mM Cl_o), whereas the Goldman-Hodgkin-Katz equation predicted a $+35$ -mV shift. This suggested that the current was carried mainly by Cl but that other ions also contributed.

For comparison, we also measured the reversal potential of the Ca-activated Cl current $I_{\text{Cl}2}$. This current is activated by hyperpolarization, exhibits an outwardly rectifying instantaneous I - V relationship, and is dependent on influx of extracellular Ca (9, 21). $I_{\text{Cl}2}$ and I_{IN} clearly have different properties. After correcting for liquid junction potentials, the reversal potential of $I_{\text{Cl}2}$ shifted $+30$ mV (from -43 mV to -13 mV) upon changing from 90 mM $[\text{Cl}]_o$ to 23 mM $[\text{Cl}]_o$ (Fig. 3, *D-F*), which was very close to that predicted for a pure Cl conductance.

These data (Fig. 3, *A-F*) suggest that I_{IN} is only partly carried by Cl ions. We then examined the reversal potential of the BAPTA-insensitive component of I_{IN} . After BAPTA injection, changing extracellular Cl from 90 mM (Fig. 3*G*) to 23 mM (Fig. 3*H*) decreased the amplitude of the currents slightly but had little effect on the reversal potential (Fig. 3*I*). The absence of the effect of $[\text{Cl}]$ on the reversal potential indicated that the BAPTA-insensitive component of I_{IN} was not carried by Cl. However, the outward tail current was decreased by decreasing external $[\text{Cl}]$ (Fig. 3*I*), suggesting that there is some outward Cl current present.

It was difficult to obtain perfect I - V relationships in BAPTA-injected oocytes because the BAPTA-insensitive component of I_{IN} fluctuated somewhat during the trial under these conditions (Fig. 3, *G* and *H*). Nevertheless, repeated trials in the same oocyte and in dif-

ferent oocytes provided consistent results. The data in Fig. 3 show that I_{IN} was composed of a Ca-activated Cl conductance that was blocked by intracellular BAPTA and a cation conductance.

Effects of divalent cations on I_{IN} . To determine the ionic selectivity of the BAPTA-insensitive component of I_{IN} to different divalent cations, the cells were bathed in solutions that contained 90 mM NMDG-Cl, 5 mM NMDG-HEPES, and 5 mM of the Cl salt of the indicated divalent cation. The oocyte was injected with BAPTA to block Ca-activated Cl channels ~ 10 min before the experiment. Under these conditions, there was significant hyperpolarization-activated inward current in the presence of Ca as the only small cation (Fig. 4*A*). In contrast, there was no detectable current in the presence of Ba, Cd, or Mn. Surprisingly, there was significant current in the presence of Co, Mg, or a mixture of Ca and Mg.

It should be noted that the current in the presence of 2.5 mM Mg plus 2.5 mM Ca was only slightly smaller than the current in the presence of 5 mM Mg (Fig. 4*A*, dashed line, *top*), thus ruling out the possibility that I_{IN} was the Ca-inactivated Cl current described by others (1, 39). We attempted to estimate the conductance of the channel to monovalent ions, but this was complicated by the appearance of the Ca-inactivated Cl current on removal of extracellular divalent cations.

Ca Current Through I_{IN} Stimulates a Cl Current

Although Figs. 3 and 4 show that I_{IN} was carried partly by divalent cations when they were the only

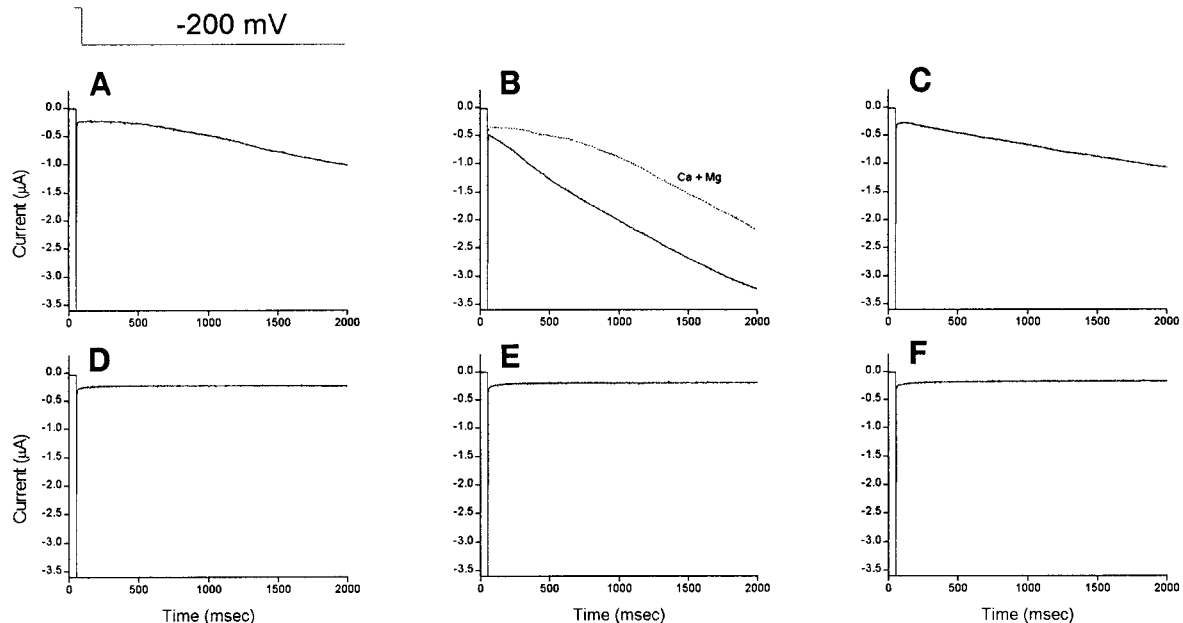


Fig. 4. Effect of divalent cations on I_{IN} . The oocytes were bathed in a solution that contained 90 mM *N*-methyl-D-glucamine (NMDG)-Cl, 5 mM NMDG-HEPES (pH 7.4), and 5 mM of the Cl salt of the indicated divalent cation and were injected with 23 nl of 50 mM BAPTA before the onset of the experiment to block Ca-activated Cl currents. Current traces are shown in response to a voltage step from -35 mV to -200 mV for cells bathed in Ca- (*A*), Mg- or Ca + Mg- (*B*), Co- (*C*), Ba- (*D*), Mn- (*E*), or Cd-containing solutions (*F*) as indicated. All traces are from the same oocyte.

permeant cations present, we wanted to determine whether Ca permeated the channel under physiological ionic conditions. We believe that Ca did permeate under physiological ionic conditions because when Ca was present in the extracellular solution, and the oocyte was not injected with BAPTA, a Ca-activated Cl current activated transiently on depolarization to +20 mV after the hyperpolarizing pulse. In Fig. 1, this current is labeled I_{OUT} , and in Fig. 2, we showed that it was blocked by BAPTA injection. This demonstrated that I_{OUT} was Ca dependent.

We hypothesized that I_{OUT} was activated by Ca entry that occurred during I_{IN} . If this is correct, we would predict that the amplitude of I_{OUT} would be related to the amplitude of I_{IN} : as more Ca entered, more Cl current would be activated. Figure 5, A and B, shows how increasing the duration of the -200-mV pulse increases both I_{IN} and I_{OUT} . As the pulse was pro-

longed, I_{IN} became larger, and the associated I_{OUT} also became larger. The observation that I_{OUT} is blocked by BAPTA and that the amplitude of I_{OUT} is related to the amplitude of the preceding I_{IN} argues that I_{OUT} is activated by Ca influx through I_{IN} .

Figure 5, C and D, shows that I_{OUT} is a Cl current because the reversal potential changes as predicted for a pure Cl current. The instantaneous I -V relationship for I_{OUT} was measured by repolarizing to +30 mV for 100 ms to activate I_{OUT} after a 2-s-long -200-mV pulse to drive Ca influx. After I_{OUT} was activated, the membrane was hyperpolarized to different potentials and the instantaneous tail current was measured (Fig. 5C). I_{OUT} had a linear instantaneous I -V relationship. On average, the reversal potential changed $+32 \pm 2.7$ mV ($n = 6$) upon changing Cl_o from 90 to 23 mM (Fig. 5D). This shift was close to the +35-mV shift predicted by the Goldman-

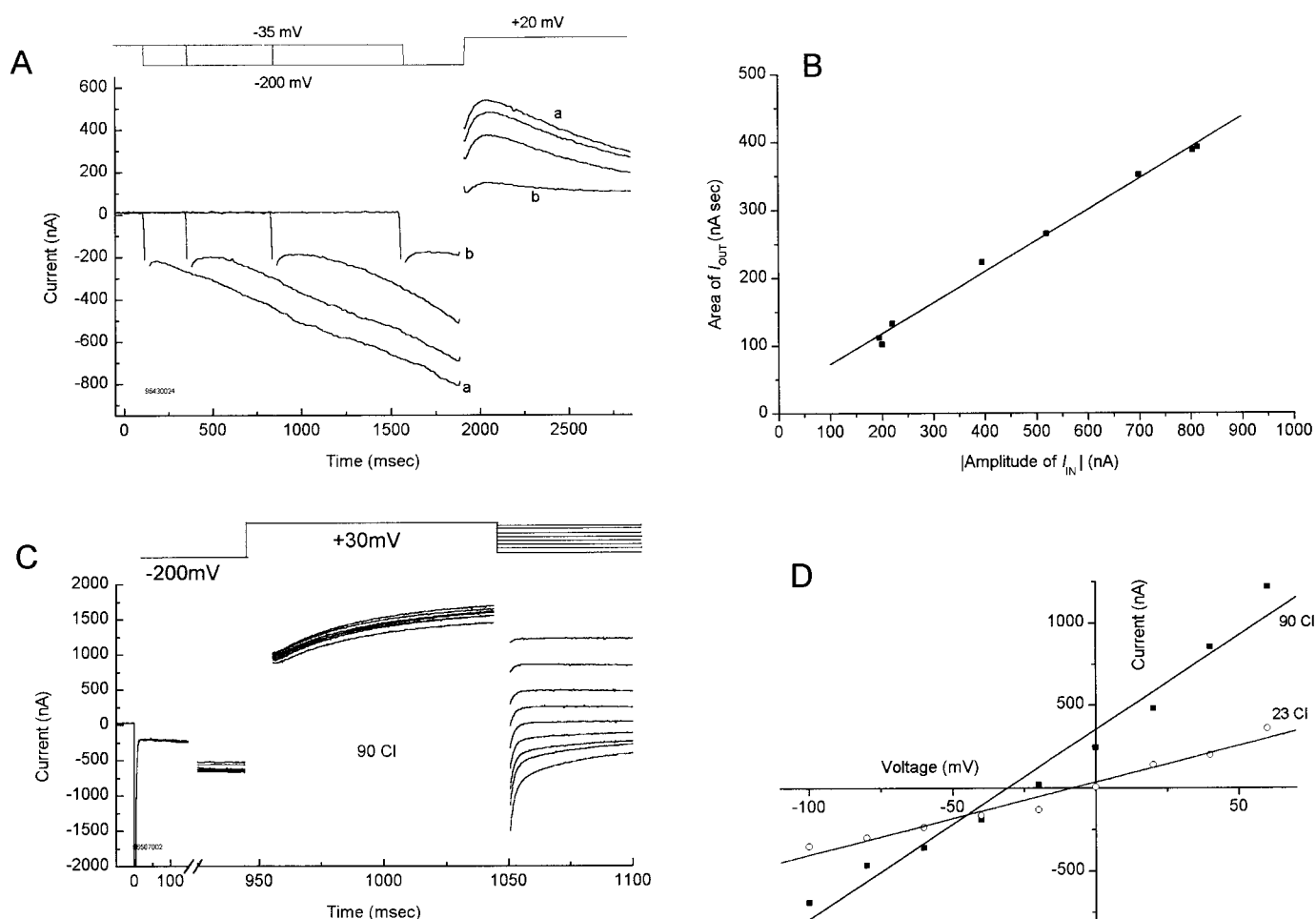


Fig. 5. Relationship of I_{OUT} to I_{IN} . A: relationship between duration of hyperpolarizing pulse and I_{OUT} . The oocyte was stepped to -200 mV for 300 ms, 1,000 ms, 1,500 ms, or 1,750 ms before stepping to +20 mV to elicit I_{OUT} . The amplitude of the inward current at the end of the -200-mV pulse correlates with the peak amplitude of I_{OUT} during the +20-mV pulse. B: relationship between the amplitude of I_{IN} at the end of the -200-mV pulse and the time integral of I_{OUT} during the +20-mV pulse for a larger set of -200-mV pulse durations. A similar relationship was observed between the peak amplitude of I_{OUT} and I_{IN} . C: instantaneous I -V relationship for I_{OUT} . The cell was hyperpolarized from a holding potential of -35 mV to -200 mV at $t = 0$. At $t = 950$ ms, the cell was stepped to +30 mV for 100 ms and then to different test potentials between +20 mV and -200 mV. The amplitude of the tail current was plotted in D for a cell bathed in 90 mM Cl (■) or 23 mM Cl (○); t , time.

Hodgkin-Katz equation. These data show that Ca entered the oocyte during the hyperpolarizing pulse and activated a Ca-activated Cl current.

Rebound Stimulation of I_{IN} After Washout of Certain Divalents

Although extracellular Cd, Ba, and Mn apparently did not permeate the channel or stimulate a Cl current, I_{IN} was greatly and transiently increased above control when these ions were washed out and replaced with a permeant divalent cation (Ca, Mg, or Co). In the experiment of Fig. 6A, the oocyte was not injected with a Ca chelator. The amplitude of I_{IN} at -180 mV was ~ 0.5 μ A. Replacement of Ca with Cd completely blocked the current. However, upon replacing Cd with Ca, I_{IN} became seven times larger and I_{OUT} became eight times larger than control. On average, I_{IN} increased 8.7 ± 0.3 -fold and I_{OUT} increased 24.2 ± 3.9 -fold ($n = 6$) within 10 s of washing out Cd. The stimulation of the current occurred both in oocytes not injected with a Ca chelator (Fig. 6A) and those injected with EGTA or BAPTA (Fig. 6B). The potentiating effect of Cd was transient and declined with a half-time of 15 ± 2 s. After 84 ± 5 s, the current stabilized at a level that was somewhat higher than before Cd.

The potentiating effect of Cd appeared to require Cd entry into the channel because the potentiating effect

of Cd was not seen when the oocyte was stepped to less negative potentials (-120 mV instead of -200 mV, Fig. 6C). Furthermore, the potentiating effect was greatly diminished if the oocyte was held at -35 mV and not hyperpolarized while Cd was present (Fig. 6D). This suggested that although Cd may not carry significant current, a small amount can enter the oocyte at these negative potentials and somehow potentiate I_{IN} when Ca is reintroduced. Similar potentiating effects were seen with washout of Mn and Ba, although this was not investigated in detail. Furthermore, washout of Cd stimulated currents in the presence of not only Ca but also Co and Mg. The mechanisms underlying this potentiation are speculative. However, the magnitude of the effect seems too large to explain by surface charge effects, and the observation that the effect occurs even in the presence of EGTA, which should chelate Cd, is paradoxical.

Stimulation of I_{IN} With Acid

Other investigators have reported that extracellular acidification produces an increase in the hyperpolarization-activated current. However, in these studies, the manner in which extracellular acidification was produced was not precisely described. When we exposed oocytes to normal Ringer solution that had been acidified by the addition of HCl, we rarely observed a

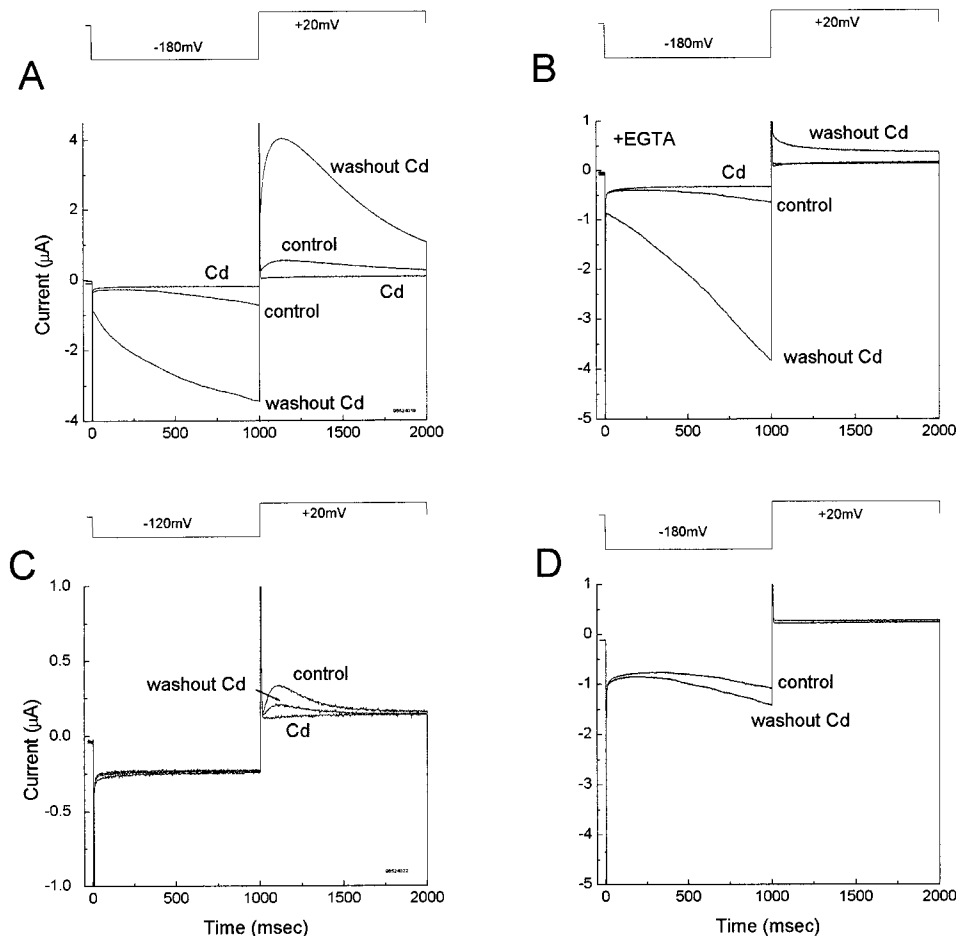


Fig. 6. Stimulatory effect of divalent cations on I_{IN} and I_{OUT} . The oocyte was voltage clamped at a -35 -mV holding potential and stepped to either -180 mV (A, B, and D) or -120 mV for 1 s followed by a 1-s pulse to $+20$ mV. The oocytes were bathed in normal Ringer solution. A: control oocyte. Three traces are superimposed: control, 2 mM Ca in the presence of 5 mM Cd, and immediately after washout of 5 mM Cd. B: EGTA-injected oocyte. The oocyte was injected with 46 nl of 100 mM EGTA (8 mM final) 10 min before the start of the experiment. C: effect of less negative hyperpolarizing pulse. The oocyte was stimulated by a voltage protocol that hyperpolarized only to -120 mV rather than -180 mV. D: effect of discontinuing stimulation in the presence of Cd. This experiment was identical to that in B, except that the oocyte was held at -35 mV during the 5-min exposure to Cd. In this case, on resumption of stimulation, the inward current was only stimulated marginally. This oocyte was injected with EGTA before the start of the experiment.

significant or reproducible increase in I_{IN} (Fig. 7A). In contrast, when we acidified the Ringer solution with a permeant, protonated acid such as acetic acid, we found that I_{IN} was stimulated dramatically. This suggested that intracellular acidification could activate

I_{IN} . This was supported by the finding that intracellular injection of HCl also stimulated this current (Fig. 7B). Isochronal I - V curves in the presence and absence of extracellular acetic acid are shown in Fig. 7C. Acidification increased the amplitude of I_{IN} at all potentials. We have previously described an intracellular acid-activated Ca influx pathway in *Chlamydomonas*, which is involved in the deflagellation response in this organism (29, 30). Some of the properties of I_{IN} (activation by acid, block by Gd, and lack of block by Co) are very similar to this *Chlamydomonas* Ca entry pathway.

I_{IN} Is Not I_{SOC}

Because I_{IN} is carried at least partly by Ca ions, we wondered whether I_{IN} could be the I_{SOC} activated by extreme hyperpolarization. To test this question, we compared some of the properties of I_{IN} and I_{SOC} in the same oocytes.

Measurement of I_{SOC} is complicated by contamination with Cl currents. In our earlier study (9), we stimulated I_{SOC} by depleting intracellular Ca stores by IP_3 injection and tried to isolate I_{SOC} from Cl currents by intracellular injection of BAPTA to block Ca-activated Cl currents. Despite the presence of 1 to 12 mM intracellular BAPTA, we found that in some cells the current that we recorded was contaminated with a Cl current. This result is shown in Fig. 8, A and B. The oocyte was bathed in a NMDG-aspartate external solution that contained 5 mM $CaCl_2$ and was periodically hyperpolarized to -150 mV for 500 ms from a holding potential of -35 mV. The oocyte was injected with 46 nl of a mixture of 1 mM 2-deoxy,3-fluoro inositol 1,4,5-trisphosphate (IP_3F) and 250 mM BAPTA to produce calculated intracellular concentrations of ~ 50 μM IP_3F and ~ 12 mM BAPTA. Inward current was measured 10 ms after the step to -150 mV. The IP_3F injection initially stimulated an inward current that peaked and decayed in <2 min. This initial component was the Ca-activated Cl tail current (9) that occurred before the BAPTA block fully developed. This initial current was followed by a slowly developing inward current. We previously suggested that this second component was I_{SOC} (9). However, we found that when we changed extracellular $[Cl]$, the inward current amplitude changed and its reversal potential shifted. This suggested that at least some of the inward current was carried by Cl ions.

We eliminated the Cl-current contamination by depleting the oocyte of intracellular Cl in addition to

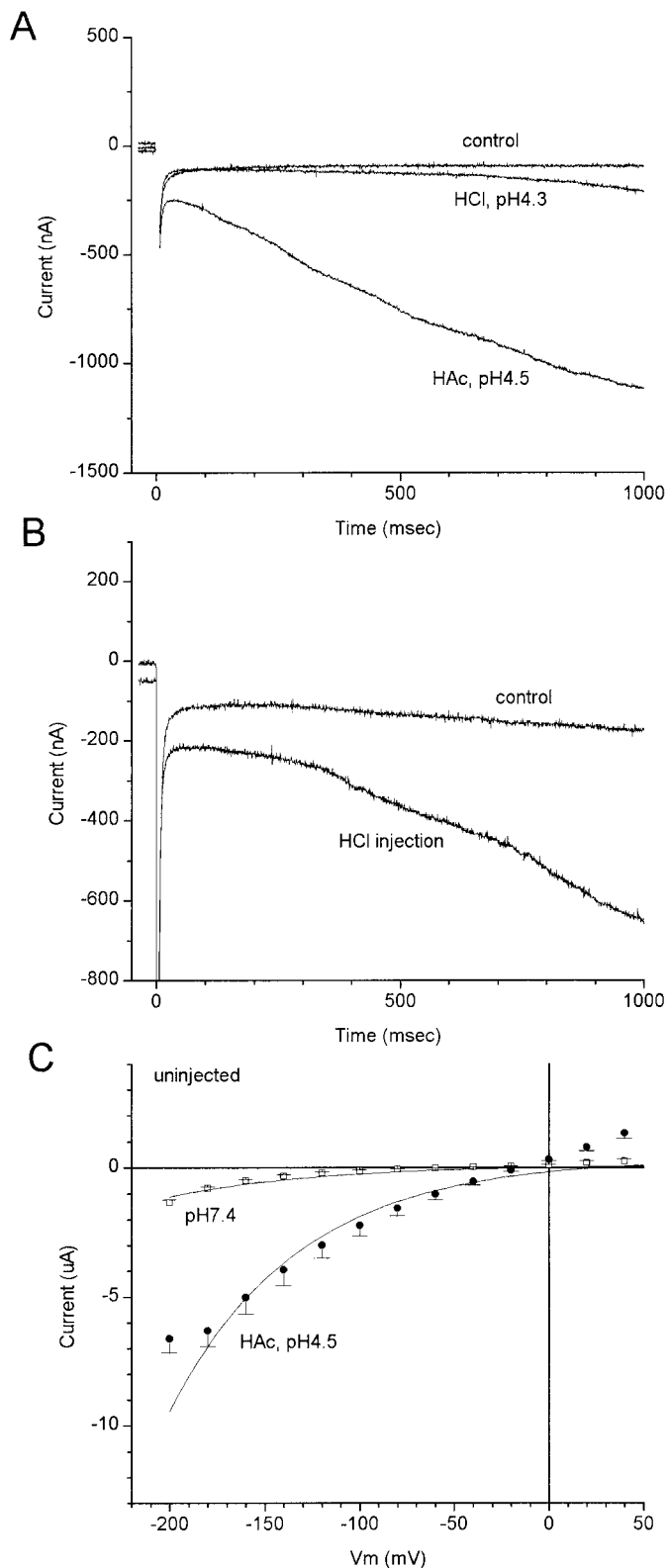


Fig. 7. Effect of acid on the hyperpolarization-activated inward current. A: an oocyte was exposed to Ringer solution that had been acidified to pH 4.3 by HCl or acidified to pH 4.5 by acetic acid (HEPES buffer was also omitted) as indicated and voltage clamped from -35 mV to -200 mV to elicit the hyperpolarization-activated inward current. B: injection of 10 nl of 10 mM HCl stimulated the hyperpolarization-activated inward current. C: changes in I - V relationship before (\square) and after (\bullet) application of acetic acid acidified Ringer solution. Currents were measured at the end of 1-s voltage steps from a holding potential of -35 mV. Data are expressed as means \pm SE from 4 oocytes each.

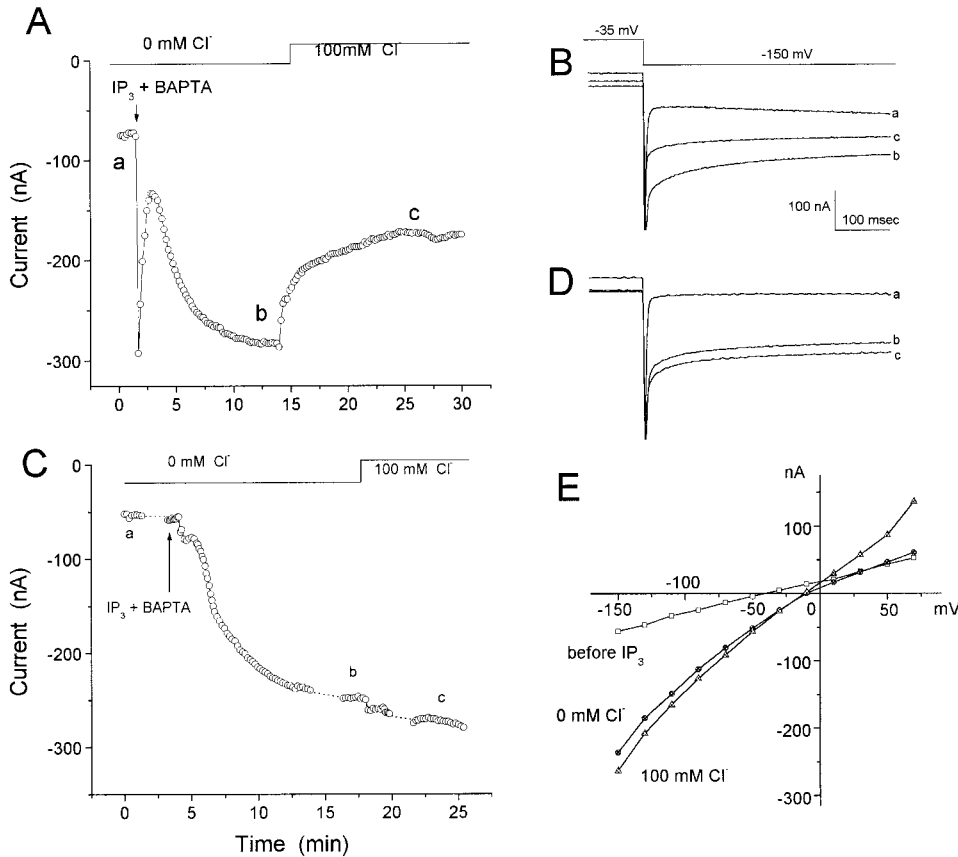


Fig. 8. Store-operated Ca current (I_{SOC}) in *Xenopus* oocytes. The oocyte was bathed in an NMDG-aspartate external solution that contained 5 mM CaCl_2 and was periodically hyperpolarized to -150 mV for 500 ms from a holding potential of -35 mV. The oocyte was injected with 46 nl of a mixture of 1 mM 2-deoxy,3-fluoro inositol 1,4,5-trisphosphate (IP_3F) and 250 mM BAPTA to produce calculated intracellular concentrations of ~ 80 μM IP_3F and ~ 20 mM BAPTA. Inward current was measured 10 ms after the step to -150 mV. A: effect of changing extracellular Cl from 0 mM to 100 mM. The letters a, b, and c refer to the times of the current traces shown in B. C: the oocyte was soaked overnight in Cl-free solution to deplete intracellular Cl. Otherwise, the experiment was performed as in A. D: current traces from experiment in C. E: I-V relationships of the currents in the experiment shown in C and D. \square , before IP_3 injection; filled circles, Cl-free solution; filled triangles, 100 mM Cl solution.

chelating intracellular Ca with ~ 12 mM BAPTA. Oocytes were depleted of Cl by soaking them overnight in a Cl-free Ringer solution (in which aspartate substituted for Cl). For recording, the oocytes were placed in a Cl-free NMDG-aspartate solution and then injected with 46 nl of 1 mM IP_3F and 250 mM BAPTA. Under these conditions, injection of IP_3F stimulated an inward current at -150 mV that developed over a period of 5–10 min (Fig. 8, C and D). After this inward current developed, changing the extracellular solution to 100 mM Cl had no effect on the inward current, which showed that the inward current was not carried by Cl ions. Figure 8E shows the I-V relationships for the current before and after IP_3F injection in the presence of 0 mM and 100 mM extracellular Cl in a Cl-depleted oocyte. The reversal potential of the current does not change, showing that the current is not carried by Cl ions.

I_{SOC} is highly Ca selective. Unlike I_{IN} , I_{SOC} was highly selective to Ca, as shown in Fig. 9, A and B. In this experiment, the oocyte was initially bathed in a solution that contained only NMDG, aspartate, HEPES, and 5 mM Ca. Changing of the extracellular solution to one that lacked Ca but contained 5 mM Mg and 0.1 mM EGTA abolished the inward current completely (Fig. 9A). The IP_3 -stimulated current strongly inwardly rectified and exhibited a reversal potential near $+25$ mV. The shape of this curve closely resembled the I-V relationship that has been reported for Ca

release-activated Ca current in other cell types (12) and I_{SOC} in oocytes (38). The inward current was strongly dependent on the extracellular Ca concentration (Fig. 9B). The dissociation constant for Ca was estimated to be 2.4 mM.

Effects of divalent cations on I_{SOC} . To investigate the effect of different cations on the store-operated Ca channel, Ca was replaced completely with different divalent cations. The replacement of Ca with Cd, Co, or Mn rapidly eliminated the current, but replacement with Ba or Sr slowly reduced the current to an intermediate level (Fig. 9C). These effects of divalent cations on I_{SOC} are different from the effects on I_{IN} .

I_{IN} is augmented by expression of TRPL, a member of a family of putative store-operated Ca channels. Our initial interest in I_{IN} developed when we noticed that this current was greatly augmented by heterologous expression of *Drosophila* TRPL (28). We were interested in TRPL because it is a member of a family of channels that has been suggested to include store-operated Ca channels (23, 40), although this remains a rather contentious issue. We found that injection of TRPL cRNA into oocytes never increased a current resembling I_{SOC} , but invariably induced a large current with characteristics very similar to I_{IN} (Fig. 10). For example, in the same batch of oocytes on the same day, in oocytes injected with 23–35 ng TRPL cRNA, the tail current at $+20$ mV from a -160 -mV pulse was 2.05 ± 0.34 μA ($n = 3$, Fig. 10C, open triangles),

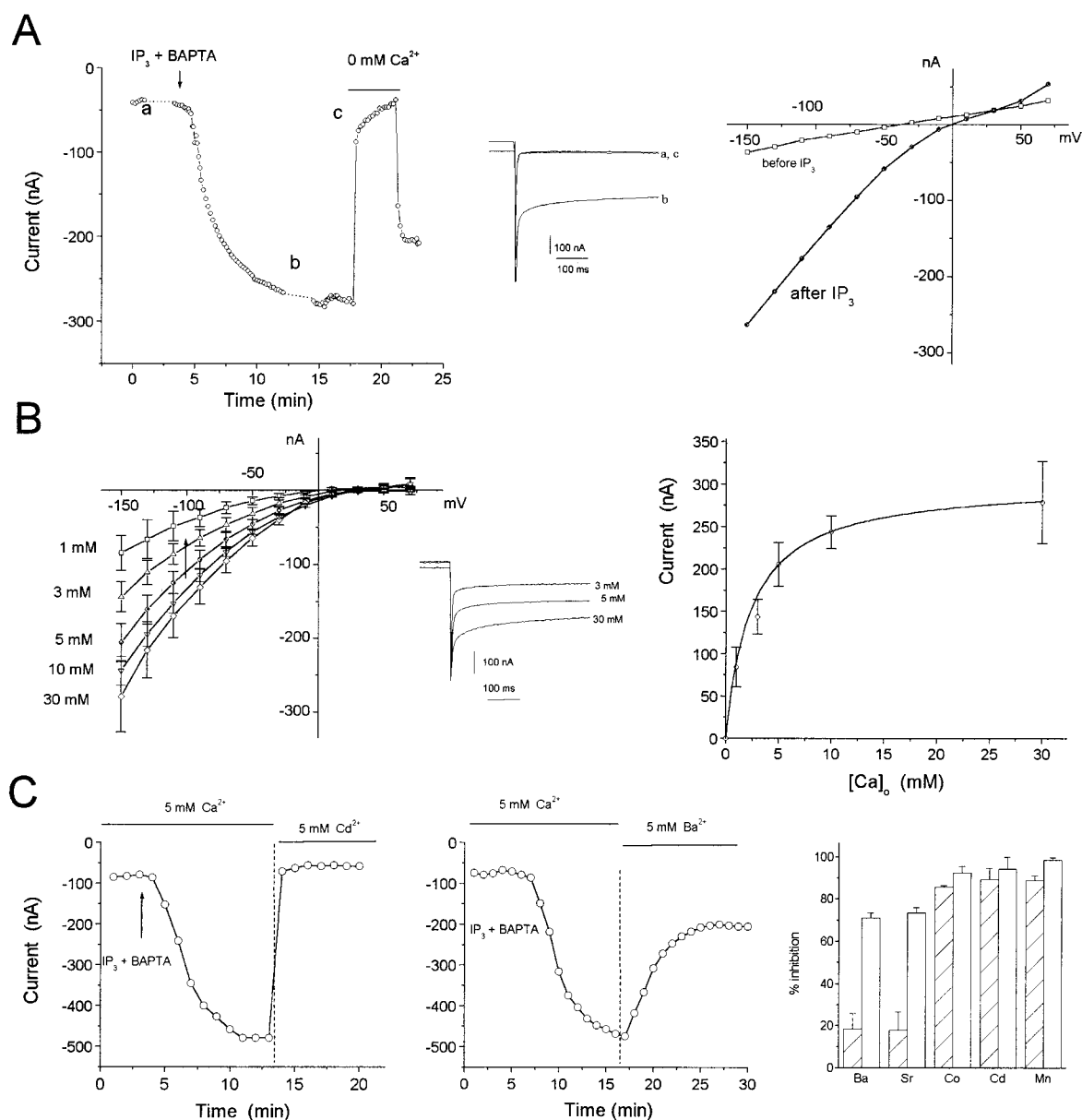


Fig. 9. Ca selectivity and effects of divalent cations on I_{SOC} . Experimental conditions are described in Fig. 8C. A: dependence of the inward current on extracellular Ca. Time course of development of I_{SOC} after injecting IP_3 and BAPTA (left). Ca was replaced with 5 mM $MgCl_2$ and 0.1 mM EGTA during the period indicated as 0 mM Ca. Current traces (middle) corresponding to a–c marked in the left panel. I–V relationship for I_{SOC} (right). B: calcium dependence of I_{SOC} . I–V relationships (left) determined as in Fig. 9A with differing Ca concentrations. The basic solution was NMDG-aspartate with 5 mM $Ca(OH)_2$ titrated with aspartic acid to pH 7.4. For other Ca concentrations, NMDG-aspartate was either increased or decreased to maintain osmolarity. Current traces (middle). Dependence of I_{SOC} amplitude on Ca concentration (right). C: effects of divalent cations on I_{SOC} . Complete exchange of the recording chamber in these experiments occurred in <20 s. Five mM Ca was replaced with 5 mM Cd (left). Five mM Ca was replaced with Ba (middle). Bar graph of inhibition of I_{SOC} by different divalent cations after 1 min of exposure (hatched bars) and after 10 min of exposure (open bars).

whereas the tail current was about seven times smaller in uninjected oocytes with a pulse of $0.3 \pm 0.2 \mu A$ ($n = 4$, Fig. 10C, closed squares). The waveform of the current and the shape of the activation curve were virtually identical for the control and TRPL oocytes, but the activation curve was shifted dramatically to the right on the voltage axis for the TRPL oocytes (Fig. 10C). We hypothesize that the I_{IN} -like current stimulated by

TRPL expression is due to upregulation of the endogenous I_{IN} current.

DISCUSSION

This manuscript characterizes a strong hyperpolarization-activated current that is present in *Xenopus* oocytes. The current, termed I_{IN} , is activated only at

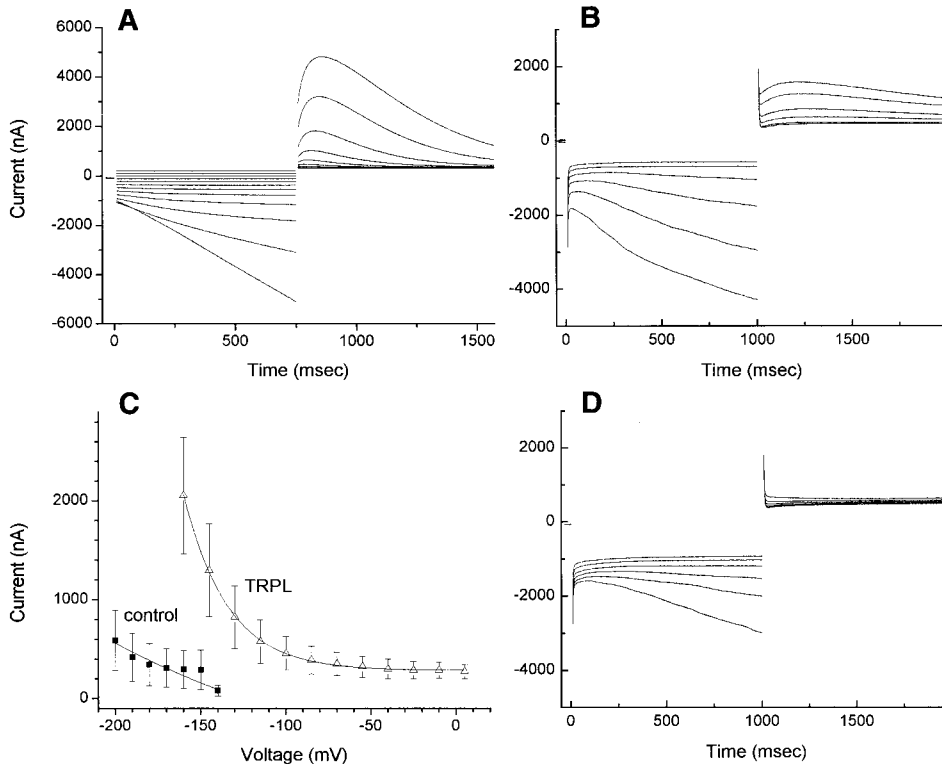


Fig. 10. Effect of heterologous expression of transient receptor potential-like gene product (TRPL) on I_{IN} . The oocytes were injected 4–6 days earlier with 23–35 ng of TRPL cRNA. A and C: the experimental details are the same as Fig. 1. I_{IN} and I_{OUT} in TRPL-expressing oocyte (A). Note that the maximum negative potential tested is -160 mV, compared with -200 mV for uninjected oocytes in Fig. 1. C: average tail current amplitudes for control (■) and TRPL-expressing (△) oocytes recorded on the same day from the same batch of oocytes. The control oocytes were the same as those shown in Fig. 1. B and D: I_{IN} and I_{OUT} in another TRPL-expressing oocyte before (B) and after (D) injecting 23 nl of 50 mM BAPTA. The experimental conditions were the same as Fig. 2.

potentials negative to -150 mV in normal oocytes, but is apparent at more positive potentials in oocytes that express the TRPL ion channel. This current is composed of two components: an inward current that is carried partly by Ca and Mg ions under physiological conditions, and a Ca-activated Cl current stimulated by Ca entering with the inward cation current. These two components can be separated by injection of BAPTA to block the Ca-activated Cl current. The inward current that remains after injection of BAPTA is not a Cl current because the current amplitude and reversal potential are independent of extracellular [Cl]. However, the observation that the conductance in the outward direction increases with increasing internal [Cl] (Fig. 3I) suggests that the current may also have a Cl component or is Cl sensitive. The current is present in solutions that contain only Ca, Mg, and Co as permeable cations but is not present in solutions that contain Ba, Mn, or Cd. It was not possible to determine the monovalent cation permeability of the channel because removing extracellular divalent cations produced a large increase in membrane conductance due to activation of the Ca-inactivated Cl current (38).

Characteristics of the Hyperpolarization-Activated Current

The complex composition of the hyperpolarization-activated current explains why this current has generated so much confusion in the literature. An inward current activated by strong hyperpolarization was first described in normal oocytes by Parker and Miledi (27). This is probably the same current we have described

here, but Parker and Miledi (27) concluded that the current was a Ca-independent Cl current because it reversed near the Cl equilibrium potential and was stimulated by removing Ca from the extracellular solution. The stimulatory effect of Ca removal (27) can be explained because they replaced Ca with Mg, which stimulates I_{IN} (Fig. 4). Parker and Miledi (27) showed that the inward current was blocked $\sim 80\%$ by 10 mM Mn at -160 mV, which is similar to what we observed.

Kowdley et al. (15) have described a slowly developing, noninactivating inward current, $I_{Cl(endo)}$, which they concluded is a voltage-dependent anion current. This current had a mean amplitude of $1.1 \mu A$ at -150 mV. They interpret this current as a Cl current because at physiological $[Cl]_o$, the reversal potential was near the Cl equilibrium potential (E_{Cl}). Furthermore, the selectivity of the channel followed the lyotropic anion sequence (11). However, the reversal potential changed ~ 35 mV/10-fold change in $[Cl]_o$, which was less than expected for a pure Cl conductance, suggesting that other conductances also contributed. The current was not affected by replacing Na with NMDG but was augmented by removing Ca. This can again be explained by the fact that their solutions contained Mg. $I_{Cl(endo)}$ resembled a current that is seen in oocytes injected with phospholemman cRNA (24). The current induced by phospholemman expression differed in some ways from the endogenous current. For example, Kowdley et al. (15) found that the phospholemman-induced current was sensitive to pH and DIDS, whereas the endogenous current was not sensitive. Furthermore, the phospholemman-induced current had different activation kinetics than the endogenous

current. Because mutations in the putative pore-forming domain of phospholemman altered the activation kinetics of the hyperpolarization-activated current observed when phospholemman was expressed in oocytes, it appears that phospholemman may form a heteromer with an endogenous subunit to enhance $I_{Cl(\text{endo})}$ (15).

Shimbo et al. (34) have reported that expression of a variety of small integral membrane proteins can modify an endogenous hyperpolarization-activated inward Cl current in *Xenopus* oocytes. The endogenous current is activated only at potentials negative to -160 mV, but in oocytes expressing the minK potassium channel, phospholemman, influenza virus NB protein, or a synthetic ion channel protein SYN-C, a similar current was activated at potentials negative to -100 mV. It appears that the I - V relationship was shifted ~ 60 mV in the positive direction by expression of exogenous ion channels. The augmentation of the current was not seen with all integral membrane proteins, because the influenza virus M2 ion channel did not stimulate this current. Furthermore, the Cl selectivity of the channel appeared to differ depending on the type of channel expressed in the oocyte: the reversal potential shifted 20 mV with a 10-fold change in Cl_o for phospholemman-expressing oocytes and 10 mV for oocytes expressing NB protein. Ba reduced both the control currents and currents in expressing oocytes $\sim 70\%$. All currents were increased by decreasing extracellular pH, which agrees with our findings but disagrees with the results of Kowdley et al. (15) that show the endogenous current was pH insensitive. The differences between these investigators may reflect the methods used to acidify the extracellular solution and the ability of the extracellular pH change to ultimately change intracellular pH.

Tzounopoulos et al. (36) also reported that expression of certain membrane proteins induced a hyperpolarization-activated current. They reported that the minK potassium channel, a mutant Shaker channel exhibiting voltage-dependent charge movement but no ion permeation, an amino acid transporter, and an inward rectifier channel all induced a hyperpolarization-activated current, whereas the dopamine D2 receptor and β -galactosidase did not. This current was identified as a Cl current because it had a reversal potential near E_{Cl} , and the reversal potential changed 53 mV/10-fold change in Cl_o . The current was stimulated by reducing extracellular Ca. In the absence of extracellular Ca, Tzounopoulos et al. (36) concluded that the current behaved as a nonselective cation current because the reversal potential of the current was similar in mixtures of K, Na, and Cs. In contrast, we find that the current is not affected by replacing Na with NMDG. The difference between our conclusions and those of Tzounopoulos et al. (36) can be explained because they implicitly assumed that the Mg present in their solutions was impermeable. If Mg is permeant and monovalent cations are not permeant, the reversal potential will not change when monovalent cation concentration is changed.

In conclusion, we believe that all of these data are consistent with our interpretation that I_{IN} is an endogenous hyperpolarization-activated current that is composed of a Ca current accompanied by a Ca-activated Cl current and possibly a small inward Ca-independent Cl current.

TRPL Expression

Our study shows that expression of TRPL results in an increase in the endogenous hyperpolarization-activated current I_{IN} . There are several possible mechanisms by which TRPL could increase I_{IN} . The first possibility is that TRPL could encode a homomeric channel that is very similar to the endogenous I_{IN} channel. This possibility seems unlikely because the properties of I_{IN} differ significantly from TRPL channels expressed endogenously in *Drosophila* photoreceptor (31) and expressed heterologously in other systems (e.g., 8, 13, 14, 19, 26, 37). The second possibility is that TRPL forms heteromeric channels with endogenous subunits. The third possibility is that TRPL somehow indirectly regulates the function of endogenous channels. We presently cannot distinguish between these alternatives. However, the observation that the voltage dependence of I_{IN} is the same in native oocytes and in oocytes expressing TRPL suggests that either the properties of this channel change with expression level or that TRPL protein is a part of the channel in expressing oocytes.

Our conclusions contrast with those of Gillo et al. (6), who conclude that coexpression of TRP (transient receptor potential) plus TRPL results in the expression of a store-operated current that they call I_{dSOC} . This current was activated by depletion of intracellular Ca stores when the quantity of injected cRNA was low, but was activated constitutively and independently of store depletion when large amounts of TRPL cRNA were injected. We believe that Gillo et al. (6) may have misidentified the current stimulated by TRPL plus TRP expression. The current that they recorded was measured in the presence of 10 mM Mg and 0 mM Ca, and its waveform closely resembled I_{IN} . The current they described differs substantially from typical I_{SOC} in selectivity and kinetics (12) and also differs significantly from TRPL channels expressed endogenously in *Drosophila* photoreceptor (31). For these reasons, we think it is likely that the I_{dSOC} current described by Gillo et al. (6) is actually the endogenous I_{IN} current.

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