Activation of Different Cl Currents in *Xenopus* Oocytes by Ca Liberated from Stores and by Capacitative Ca Influx

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ABSTRACT *Xenopus* oocytes are an excellent model system for studying Ca signaling. The purpose of this study was to characterize in detail the Ca-activated Cl currents evoked by injection of inositol 1,4,5-trisphosphate (IP3) into *Xenopus* oocytes voltage-clamped with two microelectrodes. Injection of IP3 into *Xenopus* oocytes activates two different Ca-activated Cl currents. \( I_{c11} \) is stimulated rapidly (within 5 s after IP3 injection), exhibits time-dependent activation upon depolarization, a linear instantaneous IV relationship with a reversal potential near \( E_{cl} \), and is activated by hyperpolarization with a half-maximal activation voltage of \( >200 \) mV. \( I_{c12d} \) has a strongly outwardly rectifying instantaneous IV relationship with a reversal potential near \( E_{cl} \) and is activated by hyperpolarization with a half-maximal activation voltage of \( >105 \) mV. \( I_{c12d} \) cannot be activated by Ca released from stores but is activated by Ca influx. In contrast, \( I_{c11} \) can be stimulated by Ca released from intracellular Ca stores. It can also be stimulated by Ca influx through store-operated channels if the Ca driving force is increased by a hyperpolarization immediately before the depolarization that gates \( I_{c11} \) channels. The description of two currents activated by influx and Ca release from stores provides new insights into and questions about the regulation of Ca in *Xenopus* oocytes.

Key words: *Xenopus* oocytes • inositol phosphate • Cl channels • calcium • Ca channels

INTRODUCTION

The concentration of cytosolic free Ca ([Ca]i) regulates many physiological processes. These processes are controlled by receptors that activate Ca release from intracellular stores or increase Ca influx from the extracellular space (Tsien and Tsien, 1990; Pozzan et al., 1994; Clapham, 1995). Ca is mobilized from internal stores by inositol 1,4,5-trisphosphate (IP3) produced by activation of many G-protein-coupled and tyrosine kinase-coupled receptors that stimulate phospholipase C. Release of Ca from internal stores is often followed by a sustained influx of extracellular Ca (Putney, 1990; Meldolesi et al., 1991; Putney, 1992; Putney, 1993; Fasolato et al., 1994). This influx ("capacitative Ca entry") is mediated by store-operated Ca channels (SOCCs) in the plasmalemma that are apparently controlled by the level of Ca in the internal store.

The activation of a Ca influx pathway subsequent to Ca liberation from stores has been demonstrated by showing that activation of PLC-coupled receptors produces only a transient increase in cytosolic Ca concentration when the cell is bathed in zero Ca solution, but addition of extracellular Ca results in a large sustained increase in intracellular Ca (Takeamura and Putney, 1989; Putney, 1992). The Ca influx is thought to be stimulated by "depletion" of internal stores because a similar Ca influx is stimulated by other interventions that lower store Ca. Ca-ATPase inhibitors thapsigargin, cyclopiazonic acid, and BHQ as well as intracellular bis(o-aminophenoxo)ethane-N,N',N'-tetracetic acid (BAPTA) deplete Ca stores passively by preventing reuptake of Ca that leaks out (see Pozzan et al., 1994). The Ca ionophore ionomycin at low concentrations can deplete stores and activate capacitative Ca entry without directly affecting the plasma membrane (Morgan and Jacob, 1994). Store-operated Ca channels are not opened directly by IP3 or Ca, because Ca ATPase inhibitors and ionomycin do not stimulate IP3 production, and BAPTA prevents elevation of cytosolic Ca.

*Xenopus* oocytes have been a very useful model system for studying calcium signaling partly because these cells express Ca-activated Cl channels that can be used as real-time indicators of cytosolic Ca concentration (Dascal, 1987) and partly because their large size facilitates the study of spatial and temporal changes in cytosolic Ca concentrations (Girard and Clapham, 1993; Lechleiter and Clapham, 1992). For the past year, we have been using *Xenopus* oocytes as a heterologous expression system for putative store-operated Ca channels and have used the endogenous Ca-activated Cl currents as an indirect assay of cytosolic Ca concentrations. These Cl currents have been studied extensively by others (see Dascal [1987] and references in DISCUSSION). However, we found that the behavior of these currents was more complex than we anticipated and found it necessary to undertake a more detailed, quantitative
analysis of them in order to interpret our results with heterologously expressed channels. In our analysis, we have found that there are two distinct Ca-activated Cl currents in Xenopus oocytes and that one current is activated preferentially by Ca influx through store-operated channels and that the other can be activated both by Ca influx and by Ca release from internal stores. Because these currents are dually regulated by voltage and by Ca, interpretation of effects of elevation of cytosolic Ca can be complicated.

METHODS

Electrophysiological Methods

Xenopus oocytes were voltage-clamped with two-microelectrodes using a GeneClamp 500 (Axon Instruments, Foster City, CA). Electrodes were filled with 3 M KCl and had resistances of 1-2 MΩ. 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 µl 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 per 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).

Microinjection

Oocytes were injected with IP₃ or BAPTA using a Nanoject Automatic Oocyte Injector (Drummond Scientific Co., Broomall, PA). The injection pipet was pulled from glass capillary tubing in a manner similar to the recording electrodes and then broken so that it had a beveled tip with an inside diameter of <20 µm. Typically, 4.6 nl of a 10 mM solution IP₃ in H₂O was injected to give a calculated oocyte concentration of ~50 µM. The Ca concentration in this solution was not buffered, but injection of H₂O produced no change in membrane current. Usually we injected 23 nl of a 50 mM solution of K₄'BAPTA in water to give a final calculated concentration in the oocyte of 1 mM. The injection pipets were usually left impaled in the oocyte for the duration of the experiment, unless the oocyte was injected with two different solutions, in which case the first pipet was withdrawn before the second pipet was inserted.

Solutions

Normal Ringer consisted of 123 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 1.8 mM MgCl₂, 10 mM HEPES, pH 7.4, 5 mM glucose, 5 mM sodium pyruvate. Zero Ca Ringer was the same except CaCl₂ was omitted, MgCl₂ was increased to 5 mM, and 0.1 mM EGTA was added. For 10 mM Ca Ringer, CaCl₂ was increased to 10 mM.

Harvesting Eggs

Stage V-VI oocytes were harvested from adult Xenopus laevis females (Xenopus I) as described by Dascal (Dascal, 1987). Xenopus were anesthetized by immersion in Tricaine (1.5 g/liter). Ovarian follicles were removed, cut into small pieces, and digested in normal Ringer with no added calcium containing 2 mg/ml collagenase type IA (Sigma Chemical Co., St. Louis, MO) for 2 h at room temperature. The oocytes were extensively rinsed and placed in L15 medium (Gibco BRL, Gaithersburg, MD) and stored at 18°C. Oocytes were usually used between 1 and 6 d after isolation.

Display and Analysis of Data

For display of the figures, current transients during voltage steps were either blanked for 4 ms (e.g., see Fig. 2 B) or 4 ms of the data was replaced with the value of the current immediately before the voltage step (e.g., see Fig. 3 B). Data points are the mean and error bars are ±SEM. Each current-voltage and activation functions are calculated as described in the Methods section.
RESULTS

IP₃ Injection Activates Two Currents with Different Kinetics

Fig. 1 shows the effect of injecting 4.6 nl of 10 mM IP₃ to produce a calculated intraoocyte IP₃ concentration of ~50 μM. The standard voltage protocol for these experiments was a 1-s duration pulse from a -35 mV holding potential to +20 mV followed by a 1-s duration pulse to -120 mV. The voltage pulse under basal conditions produced only small time-independent currents (Fig. 1 A, a). However, immediately after injecting IP₃, an outward current was elicited by the +20 mV pulse (Fig. 1 A, b). This current exhibited two components, an instantaneous increase followed by a slowly developing outward current. As shown below, these two components were attributed to the same current: the instantaneous component reflected increased current (due to the increased driving force) through channels that were already open at -35 mV, and the time-dependent component reflected voltage-dependent opening of additional channels. The time-dependent component was well-fitted with two exponentials with τ = 28 and 274 ms at +20 mV. Upon repolarization to -120 mV, a tail current was observed that rapidly but incompletely inactivated with τ = 17 and 67 ms. This tail current was due to the voltage-dependent closing of channels that were open at +20 mV. This outward current and its associated tail current was named Iₐ. We believe that this current is a Ca-activated Cl current that is induced by Ca released from intracellular stores by the injected IP₃. Our reasons for this conclusion are based on the observations that Iₐ (a) was blocked by 0.5 mM niflamic acid (a Ca-activated Cl channel blocker [White and Aylwin, 1990]), (b) had a reversal potential near the Cl equilibrium potential, and (c) its induction was independent of extracellular Ca and was unaffected by extracellular La, Ba, or Mn. These data are shown below.

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A

Figure 2. Steady-state IV relationship of Iₐ. The oocyte was stepped from -60 mV to different potentials between -40 and +100 mV for 500 ms followed by a step to -120 mV for 50 ms and a 500 ms step to +80 mV as shown. (A) Steady-state IV traces before IP₃ injection. (B) Steady-state IV traces after IP₃ injection. (C) Average steady-state IV relationship for six oocytes before (open circles) and after (solid squares) IP₃ injection. The currents at the end of the first voltage pulses (at 495 ms) were plotted vs. the test potential. This curve was constructed from two different voltage protocols. The first protocol was identical to the one shown in A and B. The other was similar, except that the holding potential was -120 mV and the test voltage steps were from -120 to +80. In the voltage range where these two protocols overlapped (-40 to +80 mV) the data points were identical within <10%, so the data were pooled.

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B

C

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the form $I = I_0 + A \cdot [1 - \exp(-x/\tau)]^p$ where $p = 2$ and $\tau = 289$ ms. This inward current was not accompanied by a significant outward current at $+20$ mV using this protocol. This inward current was named $I_{c1}$. This current was also a Ca-activated Cl current, as it was blocked by 0.5 mM niflumic acid and had a reversal potential that coincided with the Cl equilibrium potential. However, this current required influx of extracellular Ca to be activated as described below.

The time course of development and decay of $I_{c1}$ and $I_{c2}$ are shown in Fig. 1 B. In this plot, $I_{c1}$ was measured as the current at the end of the $+20$ mV pulse, and $I_{c2}$ was measured at the end of the $-120$ mV pulse. $I_{c1}$ developed fully within 30 s after injection of IP$_3$ and then declined nearly to baseline in $\sim 1.5$ min. $I_{c2}$ developed slowly and was not maximally activated until $>$5 min after IP$_3$ injection.

$I_{c1}$ is a Cl Current with a Linear Instantaneous Current-Voltage Relationship

Steady-state IV relationship. The "steady-state" IV curve for $I_{c1}$ was determined using the protocol shown in Fig. 2, A and B. The cell was depolarized to different command potentials for 500 ms from a holding potential of $-35$ mV before (Fig. 2 A) or immediately after (Fig. 2 B) injection of IP$_3$. The amplitude of the outward current at the end of the command pulse was

Over the next several minutes, the $I_{c1}$ outward current and its inward tail current became smaller in amplitude. Concurrent with the decrease in $I_{c1}$, a time-dependent inward current developed in response to the voltage step to $-120$ mV. This current exhibited a sigmoid onset and was fitted well by an exponential of the form $I = I_0 + A \cdot [1 - \exp(-x/\tau)]^p$ where $p = 2$ and $\tau = 289$ ms. This inward current was not accompanied by a significant outward current at $+20$ mV using this protocol. This inward current was named $I_{c1}$. This current was also a Ca-activated Cl current, as it was blocked by 0.5 mM niflumic acid and had a reversal potential that coincided with the Cl equilibrium potential. However, this current required influx of extracellular Ca to be activated as described below.

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FIGURE 5. Steady-state IV relationship for I_{Cl}. Steady state IV relationship was determined by giving a 1-s duration pulse from a holding potential of -35 to +20 mV followed by a 1-s test pulse to different potentials. (A) IV traces before IP₃ injection. (B) IV traces ~15 min after IP₃ injection. (C) IV traces after IP₃ injection and addition of 1 mM MnCl₂ to the bath. (D) Average steady-state IV relationship from four cells measured as shown in A-C. The current at 1,995 ms was plotted vs. test potential. Before IP₃ injection (solid squares), ~15 min after IP₃ injection (open circles), and after IP₃ injection with Mn added to the bath (open triangles).

plotted vs. the command potential to give the steady-state IV curve (Fig. 2 C). The curve in Fig. 2 C is the average of four oocytes, but each individual oocyte had an IV curve with the same shape. The I_{Cl} steady-state IV curve rectified strongly in the outward direction (Fig. 2 C). A problem we encountered in performing these experiments was that the amplitude of I_{Cl} changed quickly after injecting IP₃ (Fig. 1 B). To measure the IV curve quickly while I_{Cl} was not changing significantly, pulses were given at 2-s intervals. Further, to verify that the amplitude of I_{Cl} had not changed during the run, the membrane was stepped to +80 mV for 500 ms at the end of each trial to verify that I_{Cl} amplitude at this given voltage was the same for all trials. In Fig. 2 B, the amplitude of I_{Cl} at 950 ms was the same for all the trials.

The current (I) plotted in Fig. 2 C is equal to:

\[ I = N \cdot p_o \cdot \gamma \cdot (E_m - E_{rev}), \]

where \( N \) is the number of available channels, \( p_o \) is the channel open probability, \( \gamma \) is single channel conductance, \( E_m \) is membrane potential, and \( E_{rev} \) is the reversal potential of the current. To determine which of these terms might contribute to the rectification, we measured the instantaneous IV curve of I_{Cl} as shown in Fig. 3.

Instantaneous IV relationship. To measure the IV relationship, the membrane was stepped to 60 mV for 500 ms to activate I_{Cl} and then was hyperpolarized to different test potentials for 50 ms (Fig. 3, A and B). If we assume that only the driving force \((E_m - E_{rev})\) changed instantaneously, the initial amplitude at the onset of
the test pulse is equal to the current through the channels that were open at the end of the 60-mV command pulse. The instantaneous current was plotted vs. the potential of the test pulse. The interval between hyperpolarizing pulses was adjusted to the minimum required for $I_{Cl}$ to return to its fully activated amplitude (dotted horizontal line in Fig. 3 B) before the next hyperpolarizing pulse. The initial amplitude of the tail current was then plotted vs. the test potential. Before $IP_3$ injection, only very small current responses were recorded in response to the voltage pulses (Fig. 3 A). However, after $IP_3$ injection, the hyperpolarizing pulses produced large current responses (Fig. 3 B). The average instantaneous IV curve for $I_{Cl}$ recorded from four oocytes (Fig. 3 C) was essentially linear and had a reversal potential very close to the calculated Cl equilibrium potential ($-20 \text{ mV}$) (Dascal, 1987). The fact that the instantaneous IV curve was essentially linear suggested that the rectification of the steady-state IV was not due to rectification of current through open channels. This suggested that voltage-dependent gating of the channel was responsible for rectification.

**Activation curve.** To test this hypothesis, we measured the activation curve of $I_{Cl}$ using the same protocol shown in Fig. 2, A and B. In this protocol, a different number of channels were activated by stepping to different command potentials between $-40 \text{ mV} \text{ and } +100 \text{ mV}$ for 500 ms. The membrane was then repolarized to a test potential of $-120 \text{ mV}$ for 50 ms, and the initial amplitude of the tail current was measured. We also used a similar protocol with a larger command potential range of $-120 \text{ to } 80 \text{ mV}$. Because the amplitude of the tail current was always measured at the same potential, $E_n - E_{rev}$, was constant. We assumed that $\gamma$ did not change instantaneously as a function of voltage, therefore this measurement predicted the fraction of channels open at different voltages. These data showed the activation of $I_{Cl}$ increased with depolarization, but the current was not maximally activated even with command potentials as high as $+100 \text{ mV}$ (Fig. 4). The data in Fig. 4 fitted to the Boltzmann equation suggested that the half-activation potential was $+226 \text{ mV}$, although the precision of the parameters derived from this fit should be viewed with some skepticism.

Thus, the steady-state IV of $I_{Cl}$ outwardly rectified because of the voltage dependence of channel activation, but the current through the open channels had approximately a linear current-voltage relationship.

$I_{Cl,2}$ Is a Cl Current with a Strongly Outwardly Rectifying Instantaneous IV Relationship

**Steady-state IV relationship.** Fig. 5 illustrates the steady-state current-voltage relationships for oocytes before and after $IP_3$ injection. The membrane was stepped from a holding potential of $-35$ to $+20 \text{ mV}$ for 1 s before stepping to different test potentials between $-140 \text{ mV}$ and $+80 \text{ mV}$ for 1 s. The current at the end of the test pulse was plotted vs. the test potential. Under basal conditions, the cell exhibited a small outwardly rectifying current with a slope conductance between 0 mV and $-140 \text{ mV}$ of $\sim 3 \mu S$ (Fig. 5, A and D). 15 min after injection of $IP_3$ (Fig. 5 B), the cell exhibited a tilde (~)-shaped current voltage relationship: that is, the current negative to the reversal potential inwardly rectified and the current positive to the reversal potential outwardly rectified (Fig. 5 D). The current had a reversal potential near the calculated Cl equilib-
rium potential. When 1 mM MnCl₂ was added to the bathing solution, the time-dependent component of ICl₂ was completely blocked. The time-independent current was partly reduced but usually remained larger than before IP₃ injection (Fig. 5, C and D). These results are consistent with the suggestion that the time-dependent component ICl₂ was a Cl current that required Ca influx to be activated.

ICl₂ consists of two components. The current that we have termed ICl₂ consisted of two components: a time-independent component and a time-dependent component. The current-voltage relationships of the time-dependent and time-independent components are plotted separately in Fig. 6. The time-independent component was measured as the current at 1,020 ms, and the time-dependent component was measured as the difference between the current at 1,995 ms and the current at 1,020 ms. The steady-state time-dependent component appeared to rectify inwardly, whereas the current-voltage relationship of the time-independent component was essentially linear. We term these two components ICl₂D for time-dependent and ICl₂I for time-independent components. As discussed below, we believe that ICl₂D is actually ICl₁ because it exhibits properties we would predict from the characterization of ICl₁.

Instantaneous IV relationship. The steady-state IV curve in Fig. 6 showed that ICl₂D inwardly rectified. To determine the cause of this rectification, we measured the instantaneous IV relationship and the activation curve for ICl₂ using tail current analysis. The instantaneous current-voltage relationships were determined before (Fig. 7 A) and ~15 min after (Fig. 7 B) injecting IP₃. In
this experiment, the membrane was hyperpolarized to -140 mV to activate $I_{Cl2}$ and then repolarized to different test potentials. During the test pulse, an outward current developed with time. This current will be described in more detail in Fig. 11, but in the present experiment the instantaneous current was measured 4 ms after the test step. The instantaneous current at the test potential reflected the current through the channels that were open at -140 mV, before any channels had time to open or close. The basal current-voltage relationship slightly outwardly rectified and reversed at -25 mV, suggesting the presence of a small basal Cl current. After injection of IP$_3$, the current increased at least 10-fold (Fig. 7 D). The current-voltage relationship strongly outwardly rectified and reversed at the Cl equilibrium potential. The time-dependent component of $I_{Cl2}$ was completely blocked by 1 mM Mn (Fig. 7 C). It was also blocked by 0.5 mM niflumic acid.

In the experiment of Fig. 7, we did not differentiate between $I_{Cl2D}$ and $I_{Cl2i}$. To determine the instantaneous IV relationship of $I_{Cl2D}$, the IV curve of the time independent component (Fig. 6 A, squares) was subtracted from the IV curve of the total $I_{Cl2}$ current (Fig. 7 D, squares). Fig. 8 A shows these curves, and Fig. 8 B shows the subtracted IV relationship. The IV relationship of $I_{Cl2}$ strongly outwardly rectified. This seemed paradoxical, because the steady-state IV inwardly rectified. To resolve this paradox, we determined its activation curve.

**Activation curve.** The activation curve for $I_{Cl2}$ is shown in Fig. 9. The activation curve for $I_{Cl2}$ was determined by hyperpolarizing to different pre-potentials to activate $I_{Cl2}$ and then repolarizing to +20 mV to measure the instantaneous outward current through the channels that were open at the end of the prepulse. Fig. 9 A shows a typical activation protocol, and Fig. 9 B shows the average activation curve for five oocytes. $I_{Cl2}$ began to activate at potentials negative to -30 mV and exhibited a half-activation potential of -105 mV. At potentials negative to -150 mV, there was typically a decrease in $I_{Cl2}$ relative to the current at -140 mV. The activation curve in Fig. 9 B shows that the steady-state IV curve in Fig. 8 inwardly rectified because the channel did not activate at potentials positive to -30 mV. Different batches of oocytes exhibited some variability in the half-activation potential (±10 mV). The reasons for this variability have not been investigated further.

$I_{Cl2D}$ and $I_{Cl2i}$ Are Ca Activated

We hypothesize that $I_{Cl2}$ is a Ca-activated Cl current that is induced by Ca released from intracellular stores and that $I_{Cl2D}$ is a Ca-activated Cl current that is induced by Ca influx from the extracellular space. As a test of the hypothesis that these currents were Ca-activated, we first injected the oocyte with 46 nl of 100 mM K$_2$-BAPTA followed ~5 min later by an injection of 23 nl of 10 mM IP$_3$. In oocytes that had been injected with BAPTA, injection of IP$_3$ failed to induce any currents (Fig. 10 A), whereas in control oocytes, IP$_3$ injection evoked both $I_{Cl2}$ and $I_{Cl2}$ in 95% of the oocytes.

We also examined the ability of BAPTA to block $I_{Cl2}$ after it had already developed in response to IP$_3$ injection. Injection of BAPTA 10 min after IP$_3$ injection had fully activated $I_{Cl2}$ invariably blocked the time-dependent component $I_{Cl2D}$ (Fig. 10 B). In this example, the

![Figure 8](image_url)
time-independent component $I_{C_{20}}$ was apparently not blocked, but this conclusion is complicated by the fact that BAPTA alone often produced an increase in inward current that we have not fully characterized.

To determine the source of the Ca responsible for activation of $I_{C_{20}}$, we bathed the cell in solution in which the $CaCl_2$ was replaced with $BaCl_2$ (Fig. 10, C and D). Under these conditions, injection of IP$_3$ still activated $I_{C_{11}}$, but $I_{C_{20}}$ was not activated. There was an increase in inward current at $-120$ mV that corresponded to $I_{C_{20}}$. Similar results were observed when $1$ mM Mn or La was added to the normal Ca-containing solution. Furthermore, addition of Mn (Fig. 7 C) or removal of Ca blocked $I_{C_{20}}$ (see Fig. 13). These results are consistent with the hypothesis that Ca influx is required for activation of $I_{C_{20}}$. Our interpretation is that IP$_3$ first stimulates Ca release from internal stores which activates $I_{C_{11}}$. The “depletion” of Ca from stores then activates capacitive Ca entry which is responsible for $I_{C_{20}}$ activation.

**Activation of $I_{C_{21}}$ by Ca Influx**

We believe that the kinetics of decline of $I_{C_{21}}$ at $+20$ mV in Fig. 1 reflects the time course of depletion of Ca stores. Fig. 1 B may give the additional impression that $I_{C_{11}}$ cannot be activated in response to Ca influx, because $I_{C_{21}}$ declines (almost) to baseline within $\approx 3$ min after IP$_3$ injection. However, this impression is incorrect. Because the steady-state current voltage relationship of $I_{C_{21}}$ outwardly rectifies, whereas Ca influx will be greatest at hyperpolarized potentials, one would expect steady-state $I_{C_{11}}$ to be small at all potentials after stores are depleted. If this reasoning is correct, we should be able to reactivate $I_{C_{11}}$ after Ca stores are depleted by first giving hyperpolarizing pulses to increase Ca influx followed by depolarizations to activate the Cl channels. To test this hypothesis, we performed the test shown in Fig. 11. In this experiment, the oocyte was injected with IP$_3$ while stimulating with a voltage protocol similar to, but different from, the protocol used in Fig. 1. In this protocol the membrane was first stepped to $-120$ mV and then to $+20$ mV. The rationale was that Ca entering the cell when the driving force for Ca entry was high at $-120$ mV could activate $I_{C_{21}}$ at $+20$ mV, a potential which would open the voltage gates for $I_{C_{11}}$. Fig. 11 A-F shows the currents $40$ s before and 40, 50, 100, 150, and $350$ s after injecting IP$_3$. At $\sim 40$ s after IP$_3$ injection, $I_{C_{21}}$ was activated exactly as described in Fig. 1. However, as $I_{C_{21}}$ declined, it was replaced with an outward current exhibiting a different waveform that grew in amplitude in concert with the development of $I_{C_{20}}$.

The time course of development of these currents is shown in Fig. 11 H, where the circles show the initial development of $I_{C_{21}}$ measured at the end of the $+20$ mV pulse, the squares show the development of $I_{C_{20}}$ at the end of the $-120$ mV pulse, and the triangles show the current at $85$ ms after the step to $+20$ mV. The inactivating outward current at $+20$ mV which peaks in $\sim 85$ ms is referred to as $I_y$. 

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**Figure 9.** Activation curve of $I_{K_{20}}$. The activation curve was determined by stepping the membrane to different prepotentials between $-160$ and $-20$ mV for 1 s from a holding potential of $-35$ mV and then stepping to a test potential of $+20$ mV for 1 s. (A) Traces showing the determination of the activation curve. (B) The current 4 ms after stepping to the test potential was plotted as a function of the prepulse. The current for each oocyte was normalized so that the current after the $-150$ mV prepulse was set to 1.0. The data between $-150$ and $-20$ mV were fitted by the Boltzmann equation: $I = 1.05/[1 + \exp(\nu 10^{-5})/15]$. 

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**Figure 10.** C and D. Under these conditions, injection of IP$_3$ still activated $I_{C_{11}}$, but $I_{C_{20}}$ was not activated. There was an increase in inward current at $-120$ mV that corresponded to $I_{C_{20}}$. Similar results were observed when $1$ mM Mn or La was added to the normal Ca-containing solution. Furthermore, addition of Mn (Fig. 7 C) or removal of Ca blocked $I_{C_{20}}$ (see Fig. 13). These results are consistent with the hypothesis that Ca influx is required for activation of $I_{C_{20}}$. Our interpretation is that IP$_3$ first stimulates Ca release from internal stores which activates $I_{C_{11}}$. The “depletion” of Ca from stores then activates capacitive Ca entry which is responsible for $I_{C_{20}}$ activation.
FIGURE 10. Effect of Ba and BAPTA on the stimulation of IcH and IcL2. (A) The oocyte was first injected with 23 nl 50 mM K4.BAPTA. This injection usually produced an increase in time-independent inward current. 5 min later, 23 nl of 10 mM IP3 was injected. The superimposed traces shown were taken 5 min after BAPTA injection and 15 min after IP3 injection. IP3 fails to stimulate IcH or IcL2. (B) Effect of BAPTA injection on IcL2. The cell was first injected with 4.6 nl 10 mM IP3. The first trace shown was taken 15 min after IP3 injection. Subsequently, 23 nl of 50 mM BAPTA was injected. The BAPTA trace was taken 5 min after BAPTA injection. The time-dependent component of IcL2 is completely blocked. (C) Effect of extracellular Ba on stimulation of IcH and IcL2. The CaCl2 in the extracellular solution was replaced with 2 mM BaCl2. At the time indicated, 4.6 nl 10 mM IP3 was injected while the cell was stimulated with a 1-s pulse to +20 mV followed by a 1-s pulse to −120 mV from a holding potential of −35 mV. Open squares, current at the end of the +20 mV pulse (IcH); open circles, current at the end of the −120 mV pulse (IcL2). (D) Current traces from the same experiment as in C. The traces a (before IP3), b (20 s after IP3), and c (400 s after IP3) correspond to the times indicated in C.

The inactivating outward current at +20 mV has characteristics of IcL+. We hypothesize that the inactivating outward current at +20 mV (IcL+) in this protocol is IcL+ because its instantaneous current-voltage relationship resembles IcL+ and because it activates with a time constant virtually identical to IcL+. These features are illustrated in Fig. 12. The instantaneous IV relationship of the inactivating outward current was determined in an oocyte previously injected with IP3 by stepping the membrane to −120 mV for 1 s to produce Ca influx, followed by a step to +80 mV for 50 ms to activate the outward current and then stepping to different potentials for measuring the instantaneous current (Fig. 12 A). Fig. 12 A begins with the +80 mV pulse. The traces shown were obtained by subtracting the currents before injecting IP3 from those after injecting IP3, thus the IV relationship reflects only IP3-activated current. Identical results were also obtained by subtracting currents in the absence and presence of Ca in the bath. The instantaneous IV relationship was nearly linear (Fig. 12 B), which is very similar to the IV relationship of IcL+ (Fig. 3 C). The small outward rectification could possibly be
FIGURE 11. An inactivating time-dependent outward current ($I_o$) develops slowly after IP$_3$ injection. The experimental design is similar to that in Fig. 1, except that the voltage protocol was reversed: a 1-s pulse to $-120$ mV was followed by a pulse to $+20$ mV from a holding potential of $-35$ mV. A-F show current traces before and at various times after IP$_3$ injection. Note the change with time of the kinetics of the outward current at $+20$ mV. At $t = 50$ s (C) the current does not inactivate. It then declines in amplitude (D) and is replaced by an inactivating outward current (F) that grows in amplitude concurrently with the development of $I_{c1-2}$ (G). Another way of displaying the data of A-F to give a more dynamic view of the change in the outward current. 40 traces are stacked together in the z-axis starting with the first trace at the back and ending with the trace at 400 s after IP$_3$ injection at the front. The x-axis is the time of the 2-s voltage pulse and the y-axis is the amplitude. (H) The time course of change in currents after IP$_3$ injection. Open circles, $I_o$ measured as the current near the end of the $+20$ mV pulse (1,950 ms); open squares, $I_{c1-2}$ measured as the current near the end of the $-120$ mV pulse (900 ms); filled triangles, peak of the transient outward current ($I_t$), measured as the current at 1,085 ms.

due to contamination of this current with $I_{c3,2}$ which may not completely deactivate during the 50 ms pulse to $+80$ mV. We have not succeeded in selectively blocking $I_{c3,1}$ or $I_{c3,2}$ so it has been impossible to evaluate the contamination of this outward current with the underlying deactivation of $I_{c3,2}$. However, the IV curve is markedly different from that of $I_{c3,2}$, suggesting that contamination is small.

The inactivating outward current activates with the same kinetics as $I_{c1-2}$. With the voltage pulse to $+20$ mV, the inactivating outward current activated with a single exponential having $\tau = 25$ ms (Fig. 12 D), which is very similar to the $\tau$ of the fast component of the activation of $I_{c3,1}$ (Fig. 12 C). The decline of the current occurred with $\tau = \sim 250$ ms, which we presume is the time constant of reduction of cytosolic Ca upon depolarization.
Effects of Extracellular Ca

“Capacitative Ca entry” is classically demonstrated by depleting intracellular Ca stores (with IP₃ or thapsigargin) in the absence of extracellular Ca and then measuring the current that is stimulated by addition of extracellular Ca. If I₃₋₂ is activated by Ca influx, we would expect it to be activated upon re-addition of Ca to the bath after injection of IP₃ in Ca-free solutions. Fig. 13 shows the effect of switching from 0-Ca Ringer to 2 mM Ca Ringer approximately 10 min after injection of IP₃. The cell was voltage clamped at −60 mV, and the holding current was measured. Switching from 0 to 2 mM Ca in oocytes that had not been injected with IP₃ usually produced no change in the holding current at −60 mV. However, as shown in Fig. 13, when the oocyte had previously been injected with IP₃, addition of Ca produced a rapid increase in inward current. The current reached a peak of −1,000 nA in several seconds and then rapidly declined to a plateau level of −250 nA, where it remained at least 10 min. To determine which currents were responsible for this inward current at −60 mV, we returned the oocyte to 0-Ca Ringer and then ran our standard voltage protocol: a 1-s pulse to −120 mV followed by a 1-s pulse to +20 mV from a holding potential of −35 mV. Fig. 13 B–E shows the current traces before and at different times after re-addition of 2 mM Ca. At 6 s after addition of Ca, a large I₃₋₂ current was recorded at −120 mV followed by I₃₋₄ at +20 mV. Both I₃₋₂ and I₃₋₄ increased transiently after addition of Ca and then declined to a lower plateau that remained relatively constant during the remainder of the recording. The time course of change of the currents (Fig. 13 F) closely approximated the time course of decline of the holding current at −60 mV (Fig. 13 A). The decline in current is presumably due to partial refilling of the Ca store by Ca influx.

Putative Store-operated Ca Current in Oocytes

Our interpretation of the data above presumes that there is a store-operated Ca current in oocytes. We have attempted to measure this current directly as shown in Fig. 14. In this experiment, we first injected IP₃ to release Ca from intracellular pools and then injected BAPTA to inhibit Ca-activated CI currents. The cell was
stimulated with 2-s duration ramp pulses from -160 to 50 mV. Under these conditions, shifting from 0-Ca to 10 mM Ca produced a shift in the ramp current (Fig. 14A). The difference in the ramp currents in the absence and presence of Ca (Fig. 14 B) resembles the store-operated Ca current $I_{\text{SR}}$ in T-lymphocytes and mast cells (Hoth and Penner, 1993; Zweifach and Lewis, 1993; Premack et al., 1994). We cannot rule out the possibility that the difference current is residual Ca-activated Cl current not blocked by BAPTA, but the fact that there is no outward current at +50 mV (Fig. 14 B) argues against this possibility. The current is blocked by La, and its amplitude is related to the extracellular [Ca] and requires prior stimulation with IP$_3$ to be observed. Furthermore, this current activates in a voltage range that explains the activation of $I_{\text{Cl2}}$.

**DISCUSSION**

**Conclusions**

*Xenopus oocytes exhibit two different Ca-activated Cl currents.* In these studies we have shown that injection of IP$_3$ into *Xenopus* oocytes activates two different Ca-activated Cl currents. $I_{\text{Cl1}}$ is stimulated rapidly (within 5 s after IP$_3$ injection), exhibits time-dependent activation upon depolarization, a linear instantaneous IV relationship with a reversal potential near $E_{\text{Cl}}$, and a curvi-

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**FIGURE 13.** Effect of changing extracellular Ca on oocyte currents. The oocyte was injected with IP$_3$ with the oocyte bathed in normal Ringer about 10 min before the beginning of the recordings shown. After the development of $I_{\text{Cl1}}$ the oocyte was shifted into 0 Ca Ringer for ~2 min. (A) Effect of re-addition of 2 mM Ca to an oocyte bathed in 0-Ca Ringer after IP$_3$ injection. The membrane potential was held at -60 mV. The same oocyte was then shifted back to 0-Ca Ringer for ~2 rain and the standard -120 to +20 mV voltage protocol (Fig. 11) was applied once every 3 s. (B) A sample current trace in response to the -120 to 20 mV protocol in 0-Ca Ringer. (C-E) Current traces recorded 6, 22, and 50 s after addition of 2 mM Ca to the bath. (F) Plot of the change in currents with time after adding 2 mM Ca. *Filled squares*, time-dependent component of $I_{\text{Cl2}}$; *open circles*, time-independent component of $I_{\text{Cl2}}$; *open triangles*, peak transient outward $I_{\text{cl}}$ current at +20 mV (measured at 1,085 ms).
Figure 14. Putative store-operated Ca current in oocytes. The oocyte was injected with IP$_3$ ~15 min and with BAPTA ~10 min before the records shown here. The oocyte was placed in 0-Ca Ringer for 2 min, and the current recorded in response to a 2-s duration ramp pulse from -160 to +50 mV. The oocyte was shifted to 10 mM Ca and the current in response to the same ramp was recorded. The difference between these two currents (bottom) is the presumed store-operated Ca influx.

I$_{Ca}$ is stimulated both by Ca released from stores and by Ca influx. We conclude that I$_{Ca}$ is stimulated by Ca released from pools for the following reasons: (a) Intracellular injection of IP$_3$ rapidly stimulates I$_{Ca}$, and the stimulation is blocked by intracellular injection of BAPTA. This suggests that I$_{Ca}$ is stimulated by Ca and not by IP$_3$ directly. (b) Injection of IP$_3$ stimulates I$_{Ca}$ even when Ba or Mn replace extracellular Ca, suggesting that the source of stimulating Ca is not Ca influx but rather release of Ca from stores. In our analysis of the properties of I$_{Ca}$ in Figs. 2–4, we presume that [Ca]$_i$ at short times after IP$_3$ injection is relatively independent of membrane potential because Ca influx through store-operated channels has not yet been activated. Furthermore, the voltage steps used to determine the activation curve were very brief and currents were measured 4 ms after the voltage steps. One would not expect cytosolic Ca to change significantly on this time scale. Thus, the curvilinear activation curve of I$_{Ca}$ reflects voltage-dependent gating of the channel and not the level of cytosolic free Ca.

In addition to activation by store Ca, I$_{Ca}$ can be stimulated by Ca influx after the stores have been depleted, provided that Ca influx is augmented by increasing the driving force for Ca entry by hyperpolarization immediately before voltage-dependent activation of I$_{Ca}$ by a depolarization. The slowly activating current upon stepping to +20 mV from -140 mV after IP$_3$ injection is I$_{Ca}$. There are several reasons why we believe that the inactivating outward current that develops with time after IP$_3$ injection upon depolarization to +20 mV from -140 mV (Fig. 11) is I$_{Ca}$: (a) The instantaneous current-voltage relationships of the inactivating outward current at +20 mV and I$_{Ca}$ are both linear. (b) Both currents activate with the same kinetics ($	au = 25$ ms). (c) The activation curve for I$_{Ca}$ predicts that more I$_{Ca}$ will activate with depolarizations to any potential positive to -140 mV (Fig. 4). In fact, Fig. 7 B shows that upon stepping from -140 mV to various potentials, inward currents are activated negative to E$_{Cl}$ whereas outward currents are activated positive to E$_{Cl}$. This interpretation differs from that of Yao and Parker (1993) (see below).

The time-independent component of I$_{Ca}$ is probably I$_{Ca}$ for the following reasons: (a) One would expect an inward current through I$_{Ca}$ channels at potentials between E$_{Cl}$ and -150 mV based on its activation curve (Fig. 4). (b) Both I$_{Ca}$ and I$_{Ca}$ have a linear current-voltage relationship (Fig. 3 C and 8 A). (c) I$_{Ca}$ and I$_{Ca}$ exhibit identical time courses in Ca-jump experiments (Figs. 13 and 14). The only experiment in possible conflict with this interpretation is that I$_{Ca}$ was apparently not blocked by internal BAPTA (Fig. 10 B). However, this experiment may be misleading because injection of BAPTA itself before IP$_3$ produces an increase in inward
current. Thus, the apparent absence of effect of BAPTA on the time-independent inward current may be due to two opposite effects. \( I_{C2D} \) is completely blocked by 0-Ca extracellular solution (Fig. 13).

\( I_{C2D} \) is stimulated by Ca influx through store-operated Ca channels. We hypothesize that \( I_{C2D} \) is preferentially activated by Ca influx through store-operated Ca channels for the following reasons: (a) \( I_{C2D} \) requires Ca for activation, as it is blocked by intracellular injection of BAPTA. (b) \( I_{C2D} \) requires Ca influx for activation, because it is blocked by addition of Ba or Mn to the extracellular solution or removal of extracellular Ca. (c) The time course of stimulation of \( I_{C2D} \) corresponds with the time course of emptying intracellular Ca stores as assessed by the decline of \( I_{C1} \). (d) The time course of activation of \( I_{C2} \) upon stepping from \(-35 \) to \(-120 \) mV reflects the time course of accumulation of Ca in the cytosol as measured by the amplitude of \( I_{C1} \) on stepping to \(+20 \) mV at different times during the hyperpolarizing step (data not shown).

The Ca concentration-jump experiment of Fig. 13 provides additional insight into the regulation of \( I_{C2D} \). When the oocyte is shifted from 0-Ca to 2 mM Ca after IP_{3} injection, the first hyperpolarization produces a large \( I_{C2} \). This declines rapidly to a steady-state level that is less than half the amplitude of the initial current. We presume that this decline reflects refilling of the Ca stores over this \(~15\)-s period and a subsequent decrease in Ca influx. When the oocyte is shifted to 10 mM Ca (data not shown), the initial \( I_{C2} \) is extremely large, but it declines even more rapidly than with 2 mM Ca and quickly disappears. However, even though \( I_{C2} \) has disappeared, \( I_{C1} \) remains very large. There are several possible explanations of this result. One possibility is that the Ca influx refills the Ca stores, and the store-operated Ca channels subsequently close completely, but \( I_{C1} \) remains elevated because of oscillatory Ca release from stores.

Store-operated Ca current. The activation of \( I_{C2} \) by hyperpolarizing steps (Fig. 9) in principle could be explained either by strictly voltage-dependent gating of the Cl channels or could result indirectly from the voltage-dependent influx and accumulation of Ca. The latter possibility seems more likely because the putative store-operated Ca current (Fig. 14) predicts the activation of \( I_{C2} \). That is, the store-operated current activates significantly only at potentials negative to \(-50 \) mV, as does \( I_{C2D} \). This current-voltage relationship for the store-operated Ca influx explains why the inactivating outward \( I_{C1} \) current is present only when the membrane is hyperpolarized to potentials negative to \(-50 \) mV before the depolarizing step (Fig. 9 A). The observation that the voltage range for activation of outward \( I_{C1} \) parallels the activation range for the putative store-operated current (Fig. 14) provides an additional argument that this current is a store-operated current. The other arguments are that this current is dependent on extracellular Ca, is blocked by La, and is present when all Ca-activated Cl currents measured by our conventional protocols are blocked by intracellular BAPTA.
Model of regulation of Ca-activated Cl channels in Xenopus oocytes. Our model for regulation of Cl channels by Ca is shown in Fig. 15. Injection of IP₃ stimulates release of Ca from stores, which rapidly stimulates Iᶜₓ₁ channels (upper panel). As the stores become depleted, store-operated Ca channels are activated which can stimulate both Iᶜₓ₁ and Iᶜₓ₂ depending on potential (lower panel). It should be noted that because we have not inhibited the Ca-ATPase, Ca uptake into the SR has not been inhibited. Thus, activation of Iᶜₓ₁ at long times after IP₃ injection could actually be due to Ca entering the cell via influx subsequently entering Ca stores and subsequently activating Iᶜₓ₁.

At present we have no explanation why store Ca is not capable of activating Iᶜₓ₂. It seems unlikely that the explanation is that Iᶜₓ₁ and Iᶜₓ₂ have different sensitivities to Ca. Approximately the same amount of Iᶜₓ₁ is activated by store Ca and by Ca influx (Fig. 11). If we assume that the amplitude of Iᶜₓ₁ is a relative measure of the amount of Ca near these channels, this suggests that approximately the same amount of Ca is present at the membrane under these two conditions. Thus, it seems that spatial and temporal aspects of Ca concentration must be important (Lechleiter and Clapham, 1992).

Voltage-gated Ca Current

The above conclusions could be compromised if Ca influx through voltage-gated Ca channels was partly responsible for activation of these Cl currents. Xenopus oocytes have been shown to have endogenous voltage-gated Ca channels (Dascal, 1987) that might be expected to contribute Ca influx to activate Cl currents. Such a Cl current (Iᶜₓ(ca)) activated by Ca influx through voltage-gated channels has been described by Barish (1983) and Miledi (1982). However, there are a number of reasons why Ca influx via voltage-gated channels is unlikely to contribute significantly to the currents described here. (a) Iᶜₓ(ca) described by Barish (1983) is at least 10-times smaller than the currents we have described here with physiological concentrations of external Ca. (b) The IV relationship for Iᶜₓ(ca) is √-shaped with a peak near 0 mV, reflecting the IV relationship of the voltage-gated Ca channels (Barish, 1983). Neither Iᶜₓ₁ nor Iᶜₓ₂ exhibit an obvious component with this shape IV relationship. (c) In the experiments on Iᶜₓ₁ in Figs. 2–4, Iᶜₓ₁ did not require extracellular Ca. Furthermore, the holding potential was −35 mV which would largely inactivate the voltage-gated Ca channels (Dascal et al., 1986). Thus, the properties of Iᶜₓ₁ activated by store Ca would not be convoluted by any contribution from Iᶜₓ(ca). (d) In principle, Ca influx via voltage-gated Ca channels could possibly confound the tail analysis of Iᶜₓ₂ if the test depolarizations after the −120 mV steps (Fig. 7) were to activate voltage-gated Ca channels (that could not be resolved from the capacitative transient) with the resulting Ca influx activating Cl channels. In fact, however, the contribution of Ca influx via voltage-gated channels is not important because the envelope-of-tails test (Hodgkin and Huxley, 1952) shows that the tail currents precisely predict the time course of Iᶜₓ₂, which would not be true if a voltage-gated Ca influx were contributing to currents recorded during the tail. Furthermore, neither 100 μM nickel nor 10 μM nifedipine, which block the endogenous T-like and L-like Ca currents in the oocyte (Barish, 1983; Dascal et al., 1986; Dascal, 1987), had any effect on the shape of the Iᶜₓ₂ IV relationship, although they both decreased the amplitude of Iᶜₓ₂ ~50%.

Relationship to Other Studies

Since the early 1980’s, a great deal has been learned about Ca signaling and Cl channels in Xenopus oocytes, largely as the result of the pioneering work of R. Miledi and I. Parker, Y. Lass and N. Dascal, and M. Berridge and R.F. Irvine and their colleagues. The ionic currents that we have described here have been observed by these investigators previously. However, the present study adds to the earlier ones by characterizing the biophysical properties of these channels in more detail to show that there are clearly two different kinds of Ca-activated Cl channels that are regulated differently.

IP₃ activates Cl currents with several components. In reading the literature on Xenopus oocytes, one can divide Cl current responses to agents that mobilize store Ca into three different groups based on their time courses and dependence on extracellular Ca. The first response is a transient current that activates and inactivates in ~2 min and is not dependent on extracellular Ca. This current is very similar to Iᶜₓ₁ and is likely to be caused by release of Ca from stores by IP₃. The second component is also independent of extracellular Ca, but develops slowly after release of store Ca. This current may be activated by a slower mobilization of store Ca under certain conditions and may also be mediated by Iᶜₓ₁. The third component is identical to Iᶜₓ₂ in that it is activated at hyperpolarizing potentials and requires extracellular Ca. Despite the fact that it is possible to identify these three kinds of responses based on time course and sensitivity to Ca, it is difficult to compare results of different investigators unambiguously, because the current responses in the various studies were often not characterized in sufficient detail either biophysically or pharmacologically. For example, current voltage relationships of currents that one might expect to be the same based on their time course and Ca sensitivity are often different.

Most publications have focussed on only one or two of these components, but all three components were observed in the same oocytes by Snyder et al. (1988),
who demonstrated that IP₃ injection into the oocyte induced an inward current at -50 mV holding potential having three components. One component called Iₐ was independent of Ca influx and activated and inactivated in ~2 min. Iₐ was also independent of Ca influx, but activated much more slowly and lasted ~10 min or more. I₂ was dependent on Ca influx and activated slowly. It would seem that the fast Ca-activated independent component corresponds to our Icₐ1 and the slow Ca-influx dependent component corresponds to our Icₐ2 but the biophysical properties of these currents were not determined (Snyder et al., 1988). Other studies usually describe two components, but the dependence of the components on Ca influx is rather variable.

In some studies, IP₃ injection or activation of receptors that activate phospholipase C induced two Ca-dependent Cl currents that did not require Ca influx. In response to IP₃ injection, a transient current developed rapidly upon IP₃ injection and this was often followed by a more slowly developing component that was frequently oscillatory in nature (Oron et al., 1985; Parker and Miledi, 1986; Berridge, 1988; Parker and Ivorra, 1991). The amplitude of the second component seemed to be quite variable in amplitude from oocyte to oocyte (Parker and Ivorra, 1991). Activation of muscarinic receptors also activated two consecutive inward Cl currents at -60 mV (D₁ and D₂ responses) that were mimicked by IP₃ injection and were independent of Ca, (Oron et al., 1985; Gillo et al., 1987). There does not seem to be agreement about the shape of the IV relationships of the currents induced by IP₃ or receptor activation. The currents have been described as both weakly outwardly rectifying (Dascal et al., 1984; Oron et al., 1985) and very strongly outwardly rectifying (Miledi et al., 1989). A strongly rectifying Ca-activated Cl current is also induced by injection of Ca into oocytes (Miledi and Parker, 1984).

In other studies, however, IP₃ injection or activation of PLC-coupled receptors induced two Ca-activated Cl currents, but only the first component was independent of Ca, (Parker et al., 1985; Parker and Miledi, 1987; Miledi et al., 1989; DeLisle et al., 1992; Lupu-Meiri et al., 1993; Petersen and Berridge, 1994). The second component which was called Tₐ and first described in detail by Parker et al. (1985) and Parker and Miledi (1987) requires Ca influx and is very likely the same as Icₐ2 described here. Parker et al. (1985) demonstrated that in some, but not all, native oocytes hyperpolarization produced a small transient inward current that increased with hyperpolarization and had a duration of several seconds. These authors reported that this current was frequently labile and disappeared after several minutes. A similar but much larger current was found in oocytes expressing receptors coupled to phospholipase C when the receptors were activated by agonist. Tₐ is a Cl current with a steady-state current-voltage relationship that resembles Icₐ2. The current is blocked by Mn-containing or 0-Ca extracellular solutions and is blocked by EGTA injection into the oocyte. This current is also activated in native oocytes by injection of IP₃. Petersen and Berridge (1994) have shown that LPA (via a PLC-coupled receptor), thapsigargin, and IP₃ activate currents that resemble Icₐ1 and Icₐ2 with regard to their differing dependence on Caₐ and time course. Indeed, Petersen and Berridge (1994) have presented data similar to our Fig. 13, but they seem to interpret the data in terms of a single Cl conductance rather than multiple conductances.

Stimulation of the serotonin (5-HT) (1C) receptor in oocytes also produced an initial rapid oscillatory current followed by a pronounced secondary current (Parekh et al., 1993), but separation of the two components into Caₐ-dependent and independent components was not as clear. The initial component was reduced ~35% by 0-Ca and thus may be partially dependent on Ca influx. The secondary component was blocked by 100 μM Cd and was sensitive to the concentration of extracellular Ca, suggesting that this component required Ca influx. In these same studies, injection of IP₃ produced variable responses: either an oscillatory current or a rapid current followed by a sustained secondary one. The sustained current was only partly blocked by Cd, suggesting that there may be overlapping components that are dependent on Ca influx and Ca release from stores under these conditions.

There are several possible explanations why some investigators observe an Icₐ2-like current and others do not. One explanation is that the ability to observe Icₐ2 depends on the voltage protocol used. At holding potentials positive to -60 mV, activation of Icₐ2 is small (Fig. 9). Furthermore, activation of Icₐ2 requires larger amounts of IP₃ than activation of IP₃ (unpublished data). In addition, the activation of Ca influx may require that the internal stores become sufficiently depleted. We have observed that with oocytes from some donors, Icₐ2 induction is very small (~100 nA). If such an oocyte is placed in 0-Ca solution for ~30 s and then returned to normal Ca, Icₐ2 becomes much larger (~1 μA) the instant the oocyte is returned to normal Ca. Thus, it seems that some oocytes are more efficient at recycling the Ca that is released in response to IP₃ such that the pools do not become depleted sufficiently to activate Ca influx. Some oocytes may lose cytosolic Ca more rapidly than others and this may determine the rate at which Icₐ2 develops. Another possible consideration is that oocytes may differ in the number of follicular cells adhering to the oocyte. David Clapham (personal communication) has found that collagenase treatment of oocytes does not always completely remove the follicular cell layer. Thus, it is formally possi-
ble that some of the currents we have described here are due to adhering follicular cells that are attached by gap junctions to the oocyte. At the level of a high-quality dissecting microscope, we do not see any follicular cells, but a more detailed study would be required to adequately address this question.

Boton et al. (1989) have described two different Ca-activated Cl conductances in oocytes permeabilized with A23187. When the permeabilized oocytes were placed in solutions containing low Ca, only one kinetic component was observed, but addition of >2 mM Ca induced a current with two distinct kinetic components. The IV curves for both components determined by switching to Ca-containing solution at different holding potentials or by voltage ramps were linear between 0 and -60 mV, but exhibited a decreasing conductance at more negative potentials. This is similar to the steady-state IV relationship of Icl,1 described here, but it remains uncertain how these two components relate to Icl,1 and Icl,2. The two components differed with respect to their sensitivity to Ca, block by injection of EGTA, and extracellular application of the Cl-channel blocker 9-AC. Furthermore, the fast and slow components were inactivated to differing extents by Ca via a mechanism that may involve phosphorylation by protein kinase C (Boton et al., 1990; Petersen and Berridge, 1994). The two components of the current response to 5-HT or ACh could also be separated on the basis of sensitivity to EGTA suggesting that these two components may be mediated by different channels or pathways.

Mechanisms of Icl,2 Activation

Yao and Parker (1993) have suggested that the T\textsubscript{in} current, which we propose is identical to Icl,2, arises because Ca influx triggers regenerative Ca release from IP\textsubscript{3}-sensitive stores. It is known that Ca and IP\textsubscript{3} act synergistically in stimulating Ca release from IP\textsubscript{3}-sensitive stores (Iino, 1990; Bezprozvanny et al., 1991). Yao and Parker (1993) used several pieces of data to support this hypothesis, but one important piece of evidence was that the Cl current continued to increase when the membrane was depolarized to arrest Ca influx (as in Fig. 7 B). Thus, they conclude that activating Ca must come from stores. However, this interpretation depends on their assumption that the Cl current consists of a single time- and voltage-independent component. However, we believe that the activating current upon depolarization is the time- and voltage-dependent activation of Icl,2. This point of view is supported by the observation that Icl,2 is stimulated by Ca influx even when the Ca stores have been completely depleted by thapsigargin and 0-Ca (Petersen and Berridge, 1994). It should be emphasized that this re-interpretation of the regulation of Cl currents does not suggest that regenerative Ca release from IP\textsubscript{3} sensitive pools does not exist under these conditions. However, the finding that Ca green fluorescence and Cl currents do not correlate well (Yao and Parker, 1993) suggests that regardless of the mechanisms generating the Ca transients, the Cl channels do not simply respond to bulk cytosolic Ca concentration.

Petersen and Berridge (1994) suggest that the activation of the Icl,2-like current involves a positive feedback mechanism, because the current induced by addition of Ca to store-depleted oocytes produces a current that increases explosively with voltage in the range between -50 and -60 mV (see Fig. 4 C, in Petersen and Berridge, 1994). Their interpretation that this current has a positive feedback depends on their assumption that the current observed in the voltage range of -30 to 0 mV is the same current as that underlying the current in the range negative to -30 mV. However, as we have shown, the current in the range positive to -30 mV is Icl,1 whereas Icl,2 activates only negative to -40 mV. Our interpretation is that Icl,2 is a voltage-dependent current that activates steeply with hyperpolarization in this range of potentials (Fig. 9 B).

In summary, there are two Ca-activated Cl currents in Xenopus oocytes. One of these is stimulated preferentially by Ca influx through store-operated channels, whereas the other can be activated by Ca released from stores or by Ca influx.

Dedicated to my father, who taught me how to use my hands. He died the day I submitted the final version of this manuscript, June 3, 1996.

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REFERENCES

Barish, M.E. 1983. A transient calcium-dependent chloride current in the immature Xenopus oocyte. J. Physiol. (Camb.), 342:309–325.

Berridge, M.J. 1988. Inositol triphosphate-induced membrane potential oscillations in Xenopus oocytes. J. Physiol. (Camb.). 403:589–599.

Bezprozvanny, L., J. Watras, and B.E. Ehrlich. 1991. Bell-shaped calcium-response curves of Ins(1,4,5)P3 and calcium-gated channels from endoplasmic reticulum of cerebellum. Nature (Lond.). 351: 751-754.
