Calcium Current Activated by Depletion of Calcium Stores in Xenopus Oocytes

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ABSTRACT Ca\(^{2+}\) currents activated by depletion of Ca\(^{2+}\) stores in Xenopus oocytes were studied with a two-electrode voltage clamp. Buffering of cytosolic Ca\(^{2+}\) with EGTA and MeBAPTA abolished I\(_{\text{Cl(Ca)}}\) and unmasked a current in oocytes that was activated by Ino\(_3\) or ionomycin in minutes and by thapsigargin or the chelators themselves over hours. At \(-60\) mV in 10 mM extracellular CaCl\(_2\) the current was typically around \(-90\) or \(-160\) nA in oocytes loaded with EGTA or MeBAPTA, respectively. This current was judged to be a Ca\(^{2+}\)-selective current for the following reasons: (a) it was inwardly rectifying and reversed at membrane potentials usually more positive than \(+40\) mV; (b) it was dependent on extracellular [CaCl\(_2\)] with \(K_m = 11.5\) mM; (c) it was highly selective for Ca\(^{2+}\) against monovalent cations Na\(^{+}\) and K\(^+\), because replacing Na\(^{+}\) and K\(^+\) by N-methyl-D-glucammonium did not reduce the amplitude or voltage dependence of the current significantly; and (d) Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\) currents had similar instantaneous conductances, but Sr\(^{2+}\) and Ba\(^{2+}\) currents appeared to inactivate more strongly than Ca\(^{2+}\). This Ca\(^{2+}\) current was blocked by metal ions with the following potency sequence: Mg\(^{2+}\) \(<\) Ni\(^{2+}\) \(<\) Co\(^{2+}\) \(<\) Mn\(^{2+}\) \(<\) Cd\(^{2+}\) \(<\) Sr\(^{2+}\) \(<\) Ba\(^{2+}\). It was also inhibited by niflumic acid, which is commonly used to block I\(_{\text{Cl(Ca)}}\) PMA partially inhibited I\(_{\text{CRAC}}\), and this effect was mostly abolished by calphostin C, indicating that the Ca\(^{2+}\) current is sensitive to protein kinase C. These results are the first detailed electrophysiological characterization of depletion-activated Ca\(^{2+}\) current in nondialyzed cells. Because exogenous molecules and channels are easy to introduce into oocytes and the distortions in measuring I\(_{\text{Cl(Ca)}}\) can now be bypassed, oocytes are now a superior system in which to analyze the activation mechanisms of capacitative Ca\(^{2+}\) influx.

KEY WORDS: calcium influx • I\(_{\text{CRAC}}\) • I\(_{\text{SOC}}\) • thapsigargin • MeBAPTA

INTRODUCTION

In nonexcitable cells, intracellular calcium release mediated by activation of phosphoinositide metabolism is followed by a “capacitative calcium influx” (Putney, 1986; Berridge, 1995). Activation of this form of calcium influx is long-lasting, which is probably vital for some physiological functions, such as activation of lymphocytes (Lewis and Cahalan, 1995). Electrical currents corresponding to the calcium influx have been well characterized in mast cells and lymphocytes with whole-cell recording methods. The current has been termed I\(_{\text{CRAC}}\) (calcium release-activated calcium current)\(^1\) because it can be activated by releasing Ca\(^{2+}\) from internal stores through several mechanisms. They include physiological liberation of calcium from internal store by Ino\(_3\) and pharmacological depletion of the store calcium by inhibitors of endoplasmic reticulum Ca\(^{2+}\)-ATPase, calcium ionophores and high levels of calcium chelators (Hoth and Penner, 1992, 1993; Fasolato, 1994). I\(_{\text{CRAC}}\) is highly selective for Ca\(^{2+}\) over monovalent cations (Hoth and Penner, 1993). Single channel currents are not resolved using patch–clamp techniques because single-channel conductances appeared to be very small according to noise analysis (Hoth and Penner, 1993; Zweifach and Lewis, 1993; Lepple-Wienhues and Cahalan, 1996).

Similarly, calcium influx can be induced in Xenopus oocytes by agonists that stimulate metabolism of phosphoinositides, by Ino\(_3\) and related inositol polyphosphates, and by thapsigargin (Parker et al., 1985; Parker and Miledi, 1987; DeLisle et al., 1995; Petersen and Berridge, 1994). Xenopus oocytes are advantageous in some aspects to study activation mechanisms of the calcium influx. Their giant size facilitates many experimental manipulations. Much data have been accumulated with Xenopus oocytes as model cells to study inositol phosphate-mediated Ca\(^{2+}\) release and Ca\(^{2+}\) homeostasis. The oocytes possess a natural calcium indicator, a calcium-activated chloride current (I\(_{\text{Cl(Ca)}}\)) (Barish, 1983; Miledi and Parker, 1984), with which one can monitor Ca\(^{2+}\) release and Ca\(^{2+}\) influx conveniently. Although I\(_{\text{Cl(Ca)}}\) remains a sensitive approach to

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\(^{1}\)Abbreviations used in this paper: BAPTA, 1,2-bis(2-aminophosphonyl)ethane-N,N',N''-tetraacetic acid; MeBAPTA, 1-(2-amino-5-methylphenoxys)-2-(2-aminophenoxys)ethane-N,N',N''-tetraacetic acid; NMDG, N-methyl-D-glucammonium; Ino\(_3\), inositol 1,4,5-trisphosphate; PMA, phorbol-12-myristate-13-acetate; NR, normal Ringer; I\(_{\text{CRAC}}\), mammalian calcium release-activated calcium current; I\(_{\text{SOC}}\), Xenopus stores-operated calcium current.

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detect $\text{Ca}^{2+}$ influx, a quantitative analysis of the $\text{Ca}^{2+}$ influx has been hampered because the relation between $I_{\text{Cl}(\text{Ca})}$ and calcium influx is complex and incompletely defined (Parker and Yao, 1994). In addition, $I_{\text{Cl}(\text{Ca})}$ is subject to various modulators, including membrane voltage (Arreola et al., 1996; Hartzell, 1996) and intracellular molecules (Hilgemann, 1995).

To better study $\text{Ca}^{2+}$ influx into oocytes, we explored approaches to record the calcium influx directly. Injection of $\text{Ca}^{2+}$ chelators was found to be a simple and efficient way to unmask the $\text{Ca}^{2+}$ current by blocking the endogenous $I_{\text{Cl}(\text{Ca})}$, whereas blockers of anion currents interfered with the $\text{Ca}^{2+}$ current. This $\text{Ca}^{2+}$ current was characterized and shown to be similar in most but not all aspects to $I_{\text{CRAC}}$ described in mast cells and lymphocytes (Hoth and Penner, 1992, 1993; Premack et al., 1994; Lewis and Cahalan, 1995). The store-operated $\text{Ca}^{2+}$ current in *Xenopus* oocytes will be referred to as $I_{\text{SOC}}$ to avoid implying that is exactly the same as the previously described $I_{\text{CRAC}}$.

**MATERIALS AND METHODS**

*Xenopus laevis* were purchased from Xenopus I (Ann Arbor, MI), NASCO (Fort Atkinson, WI) and Xenopus Express (Beverly Hill, FL). Several lobes of ovaries were surgically removed from adult females anesthetized with 0.15% 3-aminobenzoic acid ethyl ester (MS-222; Sigma Chem. Co., St. Louis, MO). Oocytes at stages V and VI (Dumont, 1972) were dissected from the ovaries. They were treated with collagenase (0.5–1 mg/ml) at room temperature for 1 h in Barth’s medium, which contained (in mM): 88 NaCl, 1 KCl, 2.4 NaHCO3, 0.33 Ca(NO3)2, 0.41 CaCl2, 0.82 MgSO4, 5 HEPES, pH 7.4, supplemented with 50 µg/ml gentamicin (GIBCO BRL, Gaithersburg, MD). Oocytes were released from all external envelopes, except for the vitelline layer, by rolling them on a poly-L-lysine-coated culture dish with a fire-polished glass bar. The oocytes were maintained at 18°C in Barth’s medium.

Defolliculated oocytes were placed in a chamber of 200 µl volume and superfused with Ringer solutions. Normal Ringer solution (NR) had the composition (in mM): 95 NaCl, 1 KCl, 1 CaCl2, 1 MgCl2, 5 HEPES, titrated to pH 7.2, as indicated by a typical outwardly rectifying current, which contained (in mM): 70 MgCl2, 10 HEPES, pH 7.2, titrated to pH 7.2 with NaOH. In experiments studying $\text{Ca}^{2+}$ selectivity over monovalent cations, Ringers contained (in mM): 55 XCl, 30 CaCl2, 10 HEPES, X = Na, K, and N-methyl-d-glucamine (NMGD), titrated to pH 7.2 with NaOH, KOH, and HCl, respectively. $\text{Cl}^{-}$-free Ringer was prepared to examine the contribution of Cl$^{-}$ to currents, which contained (in mM): 50 Ca(CH3SO3)2, 55 KCH3SO3, 10 HEPES-Na, pH 7.2. In experiments comparing conductivity of divalent cations, Ringers contained (in mM): 70 MCl2, 10 HEPES-Na, pH 7.2, M = Ca, Sr, and Ba, respectively. In experiments studying dose-dependence of currents on extracellular concentrations of $\text{Ca}^{2+}$, the Ringers had fixed concentration (in mM): 10 MgCl2 and 10 HEPES-Na, pH 7.2, while CaCl2 and NaCl varied in pairs as 1 and 100, 3 and 100, 10 and 90, 30 and 60, 100, and 0 to achieve approximately matched osmolalities.

Membrane currents were measured using a conventional two-electrode voltage clamp (Axoclamp-2B; Axon Instruments, Foster City, CA). Voltage and current electrodes were pulled and filled with 3 M KCl to have a resistance between 0.5–2 MΩ. Current output was low-pass-filtered with an eight-pole Bessel filter (Frequency Devices, Inc., Haverhill, MA) at 200 Hz in most occasions and 1 kHz in recordings applying voltage-step command. Data acquisition and membrane voltage control were performed with a PC software and a data interface (pCLAMP 6.02 and Digidata 1200; Axon instruments). Digital format of current traces was exported to a technical graphics and data analysis software (Origin, Microcal Software, Inc., Northampton, MA) for curve fitting and plotting. Recordings were taken from oocytes with input resistances from 0.3 to 2 MΩ. Oocyte membrane potential was held at −60 mV. Intracellular injections were made using a pneumatically pressure ejection device (PV800; WPI, Inc., Sarasota, FL). All experiments were done at room temperature.

Thapsigargin, ionomycin, InsP3, PMA, and calphostin C were purchased from Calbiochem Novabiochem (La Jolla, CA). EGTA (>99% pure) was obtained from Fluka (Buchs, Switzerland). For comparison with EGTA, we used MeBAPTA, a derivative of BAPTA with one extra methyl group in the 5-position, because it was already available in the laboratory (Tsien, 1981; Adams et al., 1988) in larger quantity and greater confidence of purity than commercial BAPTA. Purity was a concern because Parekh and Penner (1995) reported that 10 mM BAPTA (Sigma) blocked ATP-dependent inactivation of $I_{\text{CRAC}}$ possibly by interfering with protein kinase C. In some experiments, we used BAPTA (Molecular Probes, Eugene, OR) and obtained similar results as with MeBAPTA. Niftumic acid (Aldrich, Milwaukee, WI) was dissolved in ethanol to yield a stock solution of 250 mM. Thapsigargin, ionomycin, PMA, and calphostin C were dissolved in DMSO. Calphostin C solution was prepared and injected into albino oocytes under dim light. Final DMSO concentrations did not exceed 0.1%.

**RESULTS**

**Direct Recording of $I_{\text{SOC}}$ by Abolishing $I_{\text{Cl}(\text{Ca})}$ with MeBAPTA and EGTA**

Most batches of oocytes did not have significant spontaneous $\text{Ca}^{2+}$ influx. To induce $\text{Ca}^{2+}$ influx, oocytes were incubated with 2 μM thapsigargin in $\text{Ca}^{2+}$-free Ringer for over 3 h (Petersen and Berridge, 1994). Alternatively, the $\text{Ca}^{2+}$ influx was induced by InsP3, ionomycin and $\text{Ca}^{2+}$ chelators (see below).

$\text{Ca}^{2+}$ currents are usually masked by endogenous $I_{\text{Cl}(\text{Ca})}$ in *Xenopus* oocytes (Barish, 1983; Miledi and Parker, 1984). Therefore, experiments started with different approaches to blocking $I_{\text{Cl}(\text{Ca})}$. Initially, nifumic acid, a chloride channel antagonist, was tested as a means to inhibit $I_{\text{Cl}(\text{Ca})}$ (White and Aylwin, 1990; Parekh et al., 1993). A ramp-voltage was applied periodically to allow rapid collection of I-V relations in the range of −120 to +80 mV (see Fig. 1, inset). Adding 10 mM CaCl2 to the extracellular medium elicited $I_{\text{Cl}(\text{Ca})}$ (Fig. 1 A), as indicated by a typical outwardly rectifying I-V relation and a reversal potential of −19 mV that was close to the $\text{Cl}^{-}$ equilibrium potential (Fig. 1 B, b-a). Currents were substantially reduced by 0.5 mM nifumic acid, and the I-V relation of the residual current in the presence of nifumic acid was still almost linear with a reversal potential of −20 mV (Fig. 1 C, c-d). This re-
Residual current in the presence of niflumic acid did not appear to be a $\text{Ca}^{2+}$ current, which ought to be inwardly rectifying and to reverse at much more positive potentials.

A different approach using $\text{Ca}^{2+}$ chelators was then explored. Similar ramp-voltage protocols were run regularly to monitor chronological change of I-V relation before and after injection of the buffers. Four nmol EGTA or MeBAPTA were injected at time indicated in Figs. 2 and 3, respectively, resulting in a final concentration of 4 mM assuming a 1-$\mu$l oocyte volume. Most $I_{\text{Cl(Ca)}}$ was blocked by both chelators within a minute after the injections. The reduced current was still mostly carried by $\text{Cl}^{-}$ at that time because the I-V relation and reversal potential were close to that before the injection of the chelators (Figs. 2B and 3B). Currents induced by extracellular $\text{Ca}^{2+}$ progressively decreased to less than one-thirtieth of the original peak of $I_{\text{Cl(Ca)}}$ af-

**Figure 1.** Effects of niflumic acid and I-V relation of niflumic acid-resistant current. (A) Oocytes treated with thapsigargin to induce long-term $\text{Ca}^{2+}$ influx were voltage-clamped at $-60$ mV except for a brief voltage ramp from $-120$ to $+80$ mV (inset) applied every 40 s. $\text{Ca}^{2+}$ entry was elicited by switching bath perfusion from Mg70 medium to NR supplemented with 10 mM $\text{CaCl}_2$ (solid bar). 0.5 mM niflumic acid was present when indicated (hollow bar). Current traces (a to d) in response to the ramp-voltage command are plotted, respectively, in B and C. The leak-subtracted current traces (b-a and c-d) in B and D are drawn thicker. The zero current level is indicated next to each current trace in this and the following figures.

**Figure 2.** Direct recording of the $\text{Ca}^{2+}$ current by injection of EGTA. Oocytes activated by thapsigargin were clamped at $-60$ mV and superfused with Mg70 medium. (A) The ramp-voltage shown in the inset of Fig. 1 was applied regularly at intervals of 40 s. $\text{CaCl}_2$ 10 mM in NR was applied as indicated (solid bars) to induce $\text{Ca}^{2+}$ entry. Large outward $I_{\text{Cl(Ca)}}$ traces responding to ramp voltage were truncated. 4 nmol EGTA was injected at time indicated (arrow). The $\text{Ca}^{2+}$ current was blocked by 0.1 mM $\text{La}^{3+}$ (hollow bar) at the end of recording to measure the leak current. Pairs of current traces (a to g) were subtracted to cancel leakage and plotted against membrane potential in B and C respectively. (B) I-V relation of $\text{Ca}^{2+}$ influx-induced current obtained before (b-a) and 1 min after (d-c) injection of EGTA. (C) I-V relation of $\text{Ca}^{2+}$ influx-induced current obtained at $-4$ (e-g) and 9 min (f-g) after the injection of EGTA.
ter two min post-injection. The transient phase of $I_{\text{Cl(Ca)}}$ was completely abolished after that time. The amplitude of the sustained inward current evoked by 10 mM CaCl$_2$ was typically around 90 nA at $-60$ mV. IV relations of the leak-subtracted currents obtained from EGTA- and MeBAPTA-loaded oocytes were plotted respectively (Figs. 2 C and 3 C). Typically, IV curves of the Ca$^{2+}$ influx-induced current changed drastically $\sim$4 min after the injection, from outward to inward rectification and from negative to positive reversal potentials (Figs. 2 C, e-g and 3 C, e-g). The IV relation remained approximately similar after that time but the reversal potential of the current progressively shifted more positive, and the outward current component became even smaller at 8.5 min after the injection of the chelators (Figs. 2 C, f-g, 3 C, f-g). These results indicated that $I_{\text{Cl(Ca)}}$ was gradually abolished and a Ca$^{2+}$ current was finally revealed when Ca$^{2+}$ buffers diffused throughout the oocytes. Inhibition of $I_{\text{Cl(Ca)}}$ was dose dependent on Ca$^{2+}$ chelators. Injection of 0.4 nmol MeBAPTA per oocyte was sufficient to totally block transient $I_{\text{Cl(Ca)}}$ evoked by 10 mM CaCl$_2$, yet the current reversed at $+18$ to $-12$ mV with prominent outward current component at positive membrane potentials ($n = 6$), indicating contamination by $I_{\text{Cl(Ca)}}$. More than 1 nmol MeBAPTA and EGTA per oocyte was required to abolish $I_{\text{Cl(Ca)}}$ completely (1 mM final assuming 1 µl oocyte volume).

MeBAPTA and EGTA showed some differences. The Ca$^{2+}$ current increased by 140 ± 20% ($n = 5$) $\sim$10 min after the injection of MeBAPTA (Fig. 3), whereas it decreased by 10 ± 3% ($n = 3$) in EGTA-injected oocytes during this period (Fig. 2). Although larger in amplitude, the IV relation in MeBAPTA-injected oocytes was similar to that in EGTA-loaded oocytes (Figs. 2 C and 3 C), indicating the Ca$^{2+}$ current was potentiated by MeBAPTA. In addition, input resistance of the oocyte decreased from 1.04 ± 0.13 to 0.87 ± 0.13 MΩ ($n = 5$) within 10 min after injection of MeBAPTA. The leak conductance was not identified but was inhibited by extracellular Ca$^{2+}$. In contrast, the input resistance remained stable in EGTA-loaded oocytes ($n = 5$), suggesting that the leak conductance was associated with an action of MeBAPTA, rather than thapsigargin treatment.

Step-voltage commands were further used to examine the instantaneous IV relation. $I_{\text{CRAC}}$ in mast cells and lymphocytes had a transient peak on a millisecond time scale during hyperpolarization pulses, which resulted from local Ca$^{2+}$ feedback inhibition (Hoth and Penner, 1992; Zweifach and Lewis, 1995). This transient component of $I_{\text{SOC}}$ could be detected readily with a pulse-voltage protocol in thapsigargin-treated oocytes pre-loaded with EGTA. In experiments shown in Fig. 4, membrane potential was held at $-60$ mV and stepped to $+60$ mV for 100 ms before hyperpolarizing to various test potentials in increments of $-20$ mV (see Fig. 4 A, a). The instantaneous inward currents evoked by large hyperpolarizing pulses decayed and reached steady state within $\sim$20 ms (Fig. 4 B). The transient current peak and plateau current had similar IV relations (Fig. 4 C). Also the IV relations obtained with the ramp-voltage command were close to those at steady state.

**Dependence of Oocyte $I_{\text{SOC}}$ on Extracellular Ca$^{2+}$ Concentration**

We then examined dependence of the Ca$^{2+}$ current on extracellular Ca$^{2+}$ concentrations in oocytes treated
with thapsigargin and loaded with MeBAPTA. As the CaCl₂ concentration in perfusion Ringers was varied between 1 and 100 mM, the size of the Ca²⁺ current changed correspondingly (Fig. 5 A). The I-V relation of the current was measured at each concentration of CaCl₂ to ensure no I Cl(Ca) contamination occurred due to possible depletion of local Ca²⁺ buffers. Intervals between two CaCl₂ applications could be minimized as no inactivation of the current was seen at this time scale of recording. The current increased slightly after each previous application of CaCl₂ in oocytes loaded with MeBAPTA. Therefore application of CaCl₂ started from low to high concentration and then returned in the opposite direction to obtain an average value for further evaluation of dose dependence. A Michaelis-Menten function was used to fit the current amplitude obtained at each extracellular concentration of CaCl₂ assuming the oocyte I SOC pathway had no cooperative binding of Ca²⁺. The best fit yielded an apparent activation constant \(K_m = 11.5 \text{ mM for CaCl}_2\) (Fig. 5 B). A similar value of \(K_m = 10.9 \text{ mM}\) was obtained in a series of separate experiments, in which CaCl₂ was directly added to NR with a similar set of values without compensation of osmolarity.

**Ion Selectivity and Conductivity**

As the ion composition inside the oocyte was not controlled under our experimental procedures, bionic approaches (Hess et al., 1986) could not be applied to study the ion selectivity of I SOC. In addition, exact measurement of reversal potential of oocyte I SOC was not warranted in our conditions, because the I-V curve approached zero asymptotically, so that apparent reversal potential was strongly affected by choice of leak current for subtraction. One endogenous background current of monovalent cations (Iₗ) was evident in oocytes in Ca²⁺-free Ringer and was inhibited by both extracellular Ca²⁺ and Mg²⁺ (Arellano et al., 1995). Thus Ca²⁺-free Ringer was replaced by Mg70 medium to reduce the leak current induced by removal of Ca²⁺. Oocytes appeared healthy for at least 20 h in this saline lacking both monovalent cations and Ca²⁺. Resting potentials and membrane input resistance remained stable during this period. However, the leak current measured in Mg 70 medium appeared still larger than that in the presence of 10 mM or higher extracellular Ca²⁺, so that the subtracted current showed an artefactual inward current phase at potentials more positive than +30 mV in most oocytes. To reduce interference of the leak conductance, La³⁺ was used to block I SOC. The current acquired after adding La³⁺ was then taken as the leak current induced by removal of Ca²⁺. The La³⁺-sensitive difference current might thus contain residual Iₗ that was not blocked by Ca²⁺. In this case, the current would reverse at a potential less positive than +30 mV as marked under the current traces in A d. 

**Figure 4.** Transient I SOC induced by membrane hyperpolarization. (A) Oocytes had been activated by thapsigargin and injected with EGTA. (a) The membrane voltage was stepped from the holding potential −60 mV to +60 mV for 100 ms, followed by hyperpolarizing steps in increments of −20 mV to −120 mV final. (b) Current was recorded in Ca70 medium containing (in mM) 70 CaCl₂, 10 HEPES, pH 7.2. (c) Leak current was acquired after addition of 1 mM LaCl₃ into Ca70 medium. (d) La³⁺-sensitive calcium current was obtained as the difference between (b and c). (B) The current in A d was expanded to show the initial current decay. (C) I-V relation of transient (+) and sustained current components (X) as marked under the current traces in A d.
Membrane currents carried by Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\) were measured at membrane potential of \(-60\) mV (Fig. 7 A). Ca\(^{2+}\) current increased slowly while Ba\(^{2+}\) current decreased with time during the perfusion. In most oocytes (69%, \(n = 26\) for total number of oocytes measured), peak amplitudes of Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\) currents were about equal. In the remaining oocytes (31% of total), Ba\(^{2+}\) current was smaller than Ca\(^{2+}\) and Sr\(^{2+}\) currents. Sr\(^{2+}\) current was smaller than Ca\(^{2+}\) current in 19% of total oocytes measured. Variability in size of Sr\(^{2+}\) or Ba\(^{2+}\) current versus Ca\(^{2+}\) current may result from their poorer buffering by Ca\(^{2+}\) chelators and greater ability to inactivate their own permeability. In the few seconds required for bath turnover, Sr\(^{2+}\) or Ba\(^{2+}\) current might have already been inactivated to various extent. The fast inactivation of Sr\(^{2+}\) and Ba\(^{2+}\) current was studied with the pulse-voltage protocol (Fig. 7, B and C). The I-V relations of instantaneous Sr\(^{2+}\) and Ba\(^{2+}\) currents induced by membrane hyperpolarization steps were monotonic with voltage (Fig. 7, B b, and C b, symbol \(\times\)) and similar to those of the Ca\(^{2+}\) current. Yet, the I-V relation obtained at the end of a 200-ms voltage pulse showed a maximum at \(-80\) mV (Fig. 7, B b and C b, symbol \(\times\)). Several hypotheses such as direct voltage dependence of blockade might account for the crossover of the current traces, but this phenomenon has not been further explored experimentally.

**Inhibitory Action on Oocyte ISOC by Metal Ions and Niflumic Acid**

Whereas Sr\(^{2+}\) and Ba\(^{2+}\) permeated this oocyte ISOC pathway readily, some transition metal ions, Ni\(^{2+}\), Co\(^{2+}\), Mn\(^{2+}\), Cd\(^{2+}\), and La\(^{3+}\) blocked the current. The inhibition was reversible, allowing effects of all metal ions to be compared in single oocytes (Fig. 8). The Ca\(^{2+}\) current was elicited by 10 mM CaCl\(_2\) as control. The oocyte ISOC was reduced by 24 ± 4% (\(n = 6\)), 26 ± 4% (\(n = 6\)), 27 ± 2% (\(n = 6\)), 65 ± 3% (\(n = 6\)) of the control by 1 mM Ni\(^{2+}\), Co\(^{2+}\), Mn\(^{2+}\), or Cd\(^{2+}\), respectively. Zn\(^{2+}\) and La\(^{3+}\) blocked ISOC completely at 1 mM, and concentrations for half-inhibition (IC\(^{50}\)) were \(\sim 40\) \(\mu\)M for Zn\(^{2+}\) (\(n = 4\)) and 0.3 \(\mu\)M for La\(^{3+}\) (\(n = 3\)), respectively. In experiments evaluating the inhibitory effect of Mg\(^{2+}\), ISOC was recorded in Mg\(^{2+}\)-free solution (in mM): (10 CaCl\(_2\), 90 NMDG-Cl, 10 HEPES-Na, pH 7.2) and Mg\(^{2+}\)-containing solution (Mg\(_2+\) medium plus 10 mM CaCl\(_2\)). Mg\(^{2+}\) had a very weak inhibitory effect on ISOC inhibiting by only 24 ± 4% at 70 mM MgCl\(_2\) (\(n = 5\)).

The action of niflumic acid on ISOC was tested because it appeared to have side effects on oocyte ISOC pathway in initial experiments. At 0.5 mM, niflumic acid inhibited ISOC with only partial reversibility (Fig. 9 A). The blocking action of niflumic acid was slow, reaching half-inhibition in \(\sim 3\) min (\(n = 6\)). Only a

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**Figure 5.** Dependence of oocyte ISOC on extracellular concentration of Ca\(^{2+}\). Ca\(^{2+}\) entry in oocytes was activated by thapsigargin. The oocyte was then injected with MeBAPTA and superfused with Mg70 medium while clamping at \(-60\) mV. (A) The Ca\(^{2+}\) current was elicited by perfusion of Ringers containing various concentrations of CaCl\(_2\). Applications of CaCl\(_2\) are indicated with solid bars with concentrations in mM. (B) Relative peak current as a function of extracellular [CaCl\(_2\)], normalized to the value at 100 mM CaCl\(_2\). The smooth curve is the best fit of the equation \(I/I_{\text{max}} = 1/(1 + K_m/[\text{CaCl}_2])\) where \(I\) is the current amplitude, \(I_{\text{max}}\) the saturating value of I at infinite [CaCl\(_2\)], and \(K_m\) is the Michaelis-Menten activation constant.
small portion of I_{SOC} recovered after washing for as long as 5 min. The blockade appeared direct for oocyte I_{SOC}, rather than due to effects on a contaminating I_{Cl(Ca)}, because the I-V relation remained similar before and after the inhibition (Fig. 9B).

**Activation of Oocyte I_{SOC} by InsP_{3}, Ionomycin and EGTA**

InsP_{3}, ionomycin, and EGTA all deplete intracellular Ca^{2+} stores by mechanisms different from thapsigargin.

We tested whether these agents can also induce oocyte I_{SOC}, as expected if the latter represents capacitative influx. Oocyte membrane potential was held at −60 mV. A ramp-voltage command similar to the inset of Fig. 1 was repetitively applied to monitor change of I-V relation before and after injection of EGTA.

The Ca^{2+} current induced by InsP_{3} was illustrated in Fig. 10. A bolus of InsP_{3} was injected at time marked by an arrow to evoke Ca^{2+} release, which was indicated by a large I_{Cl(Ca)} in Ca^{2+}-free Ringer. No significant Ca^{2+}...
influx was induced by 10 mM Ca\textsuperscript{2+} before the injection of InsP\textsubscript{3}. About 2 min after the injection of InsP\textsubscript{3}, Ca\textsuperscript{2+} influx was clearly activated as indicated by a large I\textsubscript{Cl(Ca)} when bath solution was switched from Ca\textsuperscript{2+}-free Ringer to NR with Ca\textsuperscript{2+} concentration added to 10 mM. EGTA was subsequently injected to block I\textsubscript{Cl(Ca)}. About 1 min after the injection, Ca\textsuperscript{2+} influx-induced I\textsubscript{Cl(Ca)} appeared to be almost completely abolished, because the reversal potential of the current was positive and the current showed inward rectification (Fig. 10 B, a-c). This relatively quick effect of EGTA was probably due to the local action of InsP\textsubscript{3} as the two injection pipettes were close to each other. The Ca\textsuperscript{2+} current increased by 40% in the following 6 min and outward current component was further suppressed (Fig. 10 B, b-c). The time course of Ca\textsuperscript{2+} current activation by InsP\textsubscript{3} in oocytes pre-injected with MeBAPTA is depicted in Fig. 10 C.

Ionomycin induced Ca\textsuperscript{2+} influx quickly. Ca\textsuperscript{2+} influx was not significant before bath application of ionomycin as monitored by switching from Ca\textsuperscript{2+}-free Ringer to NR with 10 mM Ca\textsuperscript{2+}. Ionomycin induced Ca\textsuperscript{2+} release as indicated by I\textsubscript{Cl(Ca)} of several \textmu A in Ca\textsuperscript{2+}-free Ringer (Fig. 11 A). A sustained Ca\textsuperscript{2+} influx activity was then recorded for longer than 10 min after washing out bath ionomycin. A typically inwardly rectifying I-V relation of the Ca\textsuperscript{2+} current was obtained after injection of EGTA (Fig. 11 B, a-b). Full activation of the Ca\textsuperscript{2+} current in oocytes pre-injected with MeBAPTA was probably due to the local action of InsP\textsubscript{3} as the two injection pipettes were close to each other. The Ca\textsuperscript{2+} current increased by 40% in the following 6 min and outward current component was further suppressed (Fig. 10 B, b-c). The time course of Ca\textsuperscript{2+} current activation by InsP\textsubscript{3} in oocytes pre-injected with MeBAPTA is depicted in Fig. 10 C.

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The leak conductance did not vary systematically during this period (Fig. 12). Little or no Ca²⁺ current was seen in the first several minutes after the injection of EGTA.

Ion replacement experiments similar to those described above were also performed to examine the Ca²⁺ currents induced by InsP₃, ionomycin and EGTA. No marked difference in ion selectivity could be detected among the Ca²⁺ currents activated by these different means. No obvious I_{SOC} was found in oocytes incubated for 20 h in Ca²⁺-free Ringer supplemented with 0.1 mM EGTA (n = 3), although the transient Ca²⁺-influx-dependent I_{Cl(Ca)} described by Petersen and Berridge (1994) was seen. When the Ca²⁺-free Ringer was temporarily replaced with normal oocyte Ringer, resting potentials were around −50 mV and input resistance was 1 MΩ.

**Modulation of I_{SOC} by Protein Kinase C**

PMA inhibited I_{CRAC} in RBL-2H3 cells (Parekh and Penner, 1995), while PMA exerted biphasic actions on Ca²⁺ influx-mediated I_{Cl(Ca)} in oocytes, characterized by an initial potentiation and a subsequent inhibition of the current (Petersen and Berridge, 1994). To determine the direct action of kinase C on Ca²⁺ influx in oocytes...
with microinjected Ca\textsuperscript{2+} chelators (Figs. 2 and 3). Compared with \(I_{\text{CRAC}}\) described formerly in mast cells and Jurkat lymphocytes (Hoth and Penner, 1992, 1993; Premack et al., 1994; Lewis and Cahalan, 1995), oocyte \(I_{\text{SOC}}\) had a similar inwardly rectifying I-V relation (Figs. 2–4), high Ca\textsuperscript{2+} selectivity over Na\textsuperscript{+} and K\textsuperscript{+} (Fig. 6), and sequence of inhibitory potency by other ions, Mg\textsuperscript{2+} << Ni\textsuperscript{2+} = Co\textsuperscript{2+} = Mn\textsuperscript{2+} << Cd\textsuperscript{2+} << Zn\textsuperscript{2+} << La\textsuperscript{3+} (<< represents about one order of magnitude difference or more) (Fig. 8). The oocyte \(I_{\text{SOC}}\) pathway had similar instantaneous conductances for Ca\textsuperscript{2+}, Sr\textsuperscript{2+}, and Ba\textsuperscript{2+}, yet Ba\textsuperscript{2+} and Sr\textsuperscript{2+} currents appeared to inactivate more strongly than Ca\textsuperscript{2+} current (Fig. 7). By contrast the \(I_{\text{CRAC}}\) pathway conducts Ca\textsuperscript{2+} about twice as well as Ba\textsuperscript{2+} and Sr\textsuperscript{2+} (Lewis and Cahalan, 1995). In addition, the dependence of \(I_{\text{SOC}}\) on extracellular CaCl\textsubscript{2} concentration had an apparent \(K_m\) = 11.5 mM in the oocytes (Fig. 5), higher than that of \(I_{\text{CRAC}}\) in mast cells (\(K_m\) = 3.3 mM; Hoth and Penner, 1993) and Jurkat lymphocytes (\(K_m\) = 2.1 mM; Premack et al., 1994). Activation of \(I_{\text{SOC}}\) by EGTA and thapsigargin in oocytes was slow, usually requiring several hours to complete (Fig. 12), which might reflect the slowness of passive leak of Ca\textsuperscript{2+} from internal stores of oocytes. Total \(I_{\text{SOC}}\) in oocytes was three to four orders of magnitude larger than that in mast cells and Jurkat cells, which was probably due merely to immense size of oocytes. In terms of current density, 200 nA current in an oocyte corresponds to 1pA pF\textsuperscript{-1} because an oocyte has a membrane capacitance of 200 nF. Thus the current density in oocytes is roughly similar to that in small mammalian cells. The gigantic size of the oocytes is an attractive feature. It allows microinjection to be easily performed. Besides, direct recording of \(I_{\text{SOC}}\) should be possible on a giant membrane patch with size of around 30 mm (Hilgemann, 1995). This may offer the opportunity to separate the plasma membrane \(I_{\text{SOC}}\) pathway from the internal Ca\textsuperscript{2+} store and to test the putative diffusible messengers directly in an excised membrane patch configuration (Randriamampita and Tsien, 1993; Kim et al., 1995).

Recently a putative store-operated current in an oocyte injected with InsP\textsubscript{3} and BAPTA was revealed (Fig. 14 of Hartzell, 1996) as the difference between currents in 0 and 10 mM Ca. Although the reversal potential could not be determined and no other analysis was presented, the amplitude and inward rectification were roughly similar to those presented here (e.g., Fig. 3, f-g and Fig. 10, b-c), suggesting that we are studying the same pathway that Hartzell (1996) first detected.

**Similarities and Differences Among Niflumic Acid, EGTA and MeBAPTA as Blockers of \(I_{\text{Ca(Ca)}}\)**

Parekh et al. (1993) reported a niflumic acid–resistant current in cell-attached membrane patches in oocytes,

\[ \text{Ca}^{2+} \text{ current was measured before and after bath application of the phorbol ester PMA. The only effect of PMA on } I_{\text{SOC}} \text{ was inhibitory in oocytes activated by ionomycin (} n = 9 \text{) or thapsigargin (} n = 19 \text{). } I_{\text{SOC}} \text{ declined monotonically with time during perfusion of PMA. The inhibitory rate increased with concentration of PMA. Typically, } I_{\text{SOC}} \text{ reduced by } 49.7 \% \text{ (Fig. 13 B) 6 min after bath perfusion of PMA } 1 \mu \text{M (Fig. 13 A). The leak conductance was simultaneously decreased but by an unknown mechanism. Calphostin C was used to confirm the inhibitory effect of PMA resulted from activation of protein kinase C (Kobayashi et al., 1989). Calphostin C was injected into oocytes to reach a final concentration of } 2 \mu \text{M and kept under room fluorescent light for more than } 0.5 \text{ h before recording started, as recommended by Bruns et al. (1991). } I_{\text{SOC}} \text{ reduced by only } 14.4 \pm 2.9 \% \text{ (} n = 5 \text{) } 6 \text{ min after bath application of PMA } 1 \mu \text{M in the calphostin C–injected oocytes, indicating that inhibitory action of PMA was mostly blocked. } I_{\text{SOC}} \text{ was } 93 \pm 15 \text{ nA (} n = 5 \text{) and } 104 \pm 14 \text{ nA (} n = 4 \text{) in calphostin C–injected and control oocytes, respectively, suggesting that } I_{\text{SOC}} \text{ was not significantly modulated by protein kinase C at the resting state.}\]
stimulated by serotonin. Similar properties of niflumic acid-resistant current were observed in this whole-cell study. This current reversed at about $-20\ \text{mV}$ and had a prominent outward current component at positive membrane potentials. These characteristics of the current were attributed to increases of both Ca$^{2+}$ and K$^+$ permeability (Parekh et al., 1993). However, Ca$^{2+}$-chelator-loaded oocytes in the present study gave quite different results. The current activated by depletion of Ca$^{2+}$ store showed little outward current, no K$^+$ permeability increase, and high calcium selectivity over monovalent cations. While the exact ionic components of niflumic acid-resistant current remain to be studied more thoroughly, we feared that niflumic acid was not a specific antagonist for I$_{\text{Cl(Ca)}}$ because this drug also inhibited oocyte I$_{\text{SOC}}$ irreversibly (Fig. 9). Niflumic acid has been reported to have multiple actions on different ionic pathways. It was used originally to block anion transporters (Cousin and Motais, 1979), but also found to affect one K current (Busch et al., 1994) expressed in the oocytes, and to block I$_{\text{CRAC}}$ in rat basophilic leukemia 2H3 cells (Reinsprecht et al., 1995).

The calcium chelators block I$_{\text{Cl(Ca)}}$ not by direct channel blockade but by binding and removing Ca$^{2+}$, so that free Ca$^{2+}$ ions appearing at calcium channel pores are closely confined without spreading to activate Cl(Ca) channels (Roberts, 1993). Ca$^{2+}$ chelation has likewise helped unmask voltage-gated Ca$^{2+}$ currents from exogenous channels in oocytes (Charnet et al., 1994). To totally saturate 4 nmol buffer, the amount typically injected into the oocytes ($B_{\text{total}}$), a sustained Ca$^{2+}$ current (I) of 200 nA should last for time $t = B_{\text{total}} \cdot z \cdot F/I = 4 \times 10^{-9} \text{mol} \cdot 2 \cdot 9.65 \times 10^4 \text{coul} \cdot \text{mol}^{-1}/(2 \times 10^{-7}\text{A}) \approx 4,000\ \text{s}$, where $z$ is the valence of Ca$^{2+}$ and $F$ is Faraday’s constant. Therefore sustained recording of I$_{\text{SOC}}$ in oocytes should be possible as long as local depletion of Ca$^{2+}$ buffers does not occur.

The slow buffer, EGTA, was found to have no effect at 1 mM on Ca$^{2+}$-activated K$^+$ current in saccular hair cells (Roberts, 1993), while a similar dose of EGTA strongly inhibited I$_{\text{Cl(Ca)}}$ in oocytes. This might arise from the smaller unitary conductance of CRAC or SOC channels and possibly larger distance between Cl(Ca) channels and SOC channels in oocytes. I$_{\text{SOC}}$ was found to be consistently larger in oocytes injected with MeBAPTA than that with EGTA. Also, in oocytes buffered with MeBAPTA, I$_{\text{SOC}}$ did not instantly level off within each exposure to high extracellular Ca$^{2+}$ (see Figs. 3, 5–9), whereas in oocytes injected with EGTA, I$_{\text{SOC}}$ immediately reached a flat plateau within each pulse (see Figs. 2 and 11). These differences might result from the difference in Ca$^{2+}$ binding kinetics of the buffers, because local feedback inhibition by Ca$^{2+}$ on SOC channels is better attenuated by BAPTA due to its faster binding rate (Zweifach and Lewis, 1995). Inhibition of tonically active protein kinase C (Parekh and Penner, 1995) does not readily explain the enhancement of I$_{\text{SOC}}$ by MeBAPTA, because calphostin C did not mimic MeBAPTA, though this kinase blocker could inhibit PMA effects (Fig. 13). The slight increase in leak current induced by MeBAPTA was not desirable especially when a prolonged recording of I$_{\text{SOC}}$ was needed. EGTA would then be the better choice in this case, because neither I$_{\text{SOC}}$ nor the membrane leak was significantly affected by this chelator.

**Relationship Between I$_{\text{SOC}}$ and Ca$^{2+}$ Influx-mediated I$_{\text{Cl(Ca)}}$ in Oocytes**

I$_{\text{Cl(Ca)}}$ has been widely used to indicate Ca$^{2+}$ influx activity in the oocytes. It is a sensitive measure of Ca$^{2+}$ influx activity, as peak I$_{\text{Cl(Ca)}}$ is more than 10 times larger than the underlying calcium influx current (see Figs. 2 and 3). However, the relation between I$_{\text{Cl(Ca)}}$ and I$_{\text{SOC}}$ is not simple. Several discrepancies are obvious. First, I$_{\text{Cl(Ca)}}$ evoked by Ca$^{2+}$ influx has an initially large transient.

**Figure 13.** Inhibition of I$_{\text{SOC}}$ by activation of protein kinase C. Oocytes were treated with ionomycin and injected with EGTA. Ca$^{2+}$ current was induced by switching bath perfusion from Mg$^{70}$ medium to Ca$^{70}$ medium repetitively as indicated by the solid bars. Bath application of PMA 1 μM (hollow bar) inhibited the Ca$^{2+}$ current. (B) An albino oocyte was activated by thapsigargin. The oocyte was injected with EGTA and calphostin C 40 min before recording started. The inhibitory effect of PMA was almost completely abolished.
component that rises and decays in hundreds of milliseconds to several seconds, followed by a relatively sustained component. Second, the Ca\(^{2+}\) entry-dependent transient I\(_{\text{Cl(Ca)}}\) is a highly nonlinear function of membrane hyperpolarization and extracellular Ca\(^{2+}\) concentration (Parker et al., 1985; Petersen and Berridge, 1994). Third, most of the Ca\(^{2+}\) influx-induced transient I\(_{\text{Cl(Ca)}}\) inactivates and recovers in about one minute. Although different mechanisms were proposed, approximately similar characteristics of the I\(_{\text{Cl(Ca)}}\) were also observed in oocytes that were either injected with InsP\(_3\) (Yao and Parker, 1993) or incubated with calcium ionophores, A23187 (Boton et al., 1989) and ionomycin (Yao, Y., unpublished data). Thus, in oocytes injected with InsP\(_3\) or stimulated with agonists, Ca\(^{2+}\) influx promoted by hyperpolarization pulses evoked a large Ca\(^{2+}\)-dependent transient I\(_{\text{Cl(Ca)}}\), \(T_{\text{in}}\) (Parker et al., 1985). \(T_{\text{in}}\) appeared to rise and decay more rapidly than the transient I\(_{\text{Cl(Ca)}}\) in thapsigargin- and the ionophore-treated oocytes. In addition, \(T_{\text{in}}\) appeared to have a different onset shape. Simultaneous recording of I\(_{\text{Cl(Ca)}}\) and Ca\(^{2+}\) fluorescence showed that \(T_{\text{in}}\) reflects InsP\(_3\)-dependent Ca\(^{2+}\)-induced Ca\(^{2+}\) release (Yao and Parker, 1993). Consistent with this, the hump component of the I\(_{\text{Cl(Ca)}}\) could be elicited by membrane depolarization in oocytes expressing voltage-gated Ca\(^{2+}\) channels together with InsP\(_3\) application (Yao and Parker, 1992). This indicated that transient I\(_{\text{Cl(Ca)}}\) does not require Ca\(^{2+}\) influx via the ISOC pathway per se. The InsP\(_3\)-dependent Ca\(^{2+}\)-induced Ca\(^{2+}\) release mechanism, however, fails to explain the transient I\(_{\text{Cl(Ca)}}\) induced by Ca\(^{2+}\) influx in thapsigargin-treated oocytes since Ca\(^{2+}\) store had been depleted, as indicated by the lack of further Ca\(^{2+}\) release in response to InsP\(_3\) (Petersen and Berridge, 1994) and ionomycin (Yao, Y., unpublished observation). To explain the supralinear relation between transient I\(_{\text{Cl(Ca)}}\) and extracellular Ca\(^{2+}\) concentration or membrane hyperpolarization, a positive feedback regulation at the level of the Ca\(^{2+}\) influx pathway has been proposed (Petersen and Berridge, 1994). However, no sign of regenerativity was seen with oocyte ISOC in this study. Besides, injection of as little as 120 pmol slow buffer EGTA (final 120 \(\mu\)M assuming 1 \(\mu\)l oocyte volume) was sufficient to totally block the transient I\(_{\text{Cl(Ca)}}\) evoked by 10 mM extracellular CaCl\(_2\) in thapsigargin-treated oocytes (Y. Yao, unpublished data). This suggests that any regenerativity lies between Ca\(^{2+}\) influx and transient I\(_{\text{Cl(Ca)}}\), rather than between stores depletion and Ca\(^{2+}\) influx.

Alternatives to regenerativity to interpret the nonlinearity of Ca\(^{2+}\) influx-induced I\(_{\text{Cl(Ca)}}\) should be considered. First, diffusion and buffering processes could contribute to the nonlinearity. Local free [Ca\(^{2+}\)] profiles are dependent on intensity of Ca\(^{2+}\) current source and Ca\(^{2+}\) buffers. Mobility, binding and dissociation kinetics and concentration of the endogenous Ca\(^{2+}\) buffers are all critical variables to shape local [Ca\(^{2+}\)]. Qualitatively, with a small Ca\(^{2+}\) influx, the endogenous buffers would be sufficient to bind and remove Ca\(^{2+}\) so that no or low free Ca\(^{2+}\) could reach Cl(Ca) channels and hence no or small I\(_{\text{Cl(Ca)}}\). With large influx of Ca\(^{2+}\), the Ca\(^{2+}\) buffers would be depleted locally, so that more free Ca\(^{2+}\) would spread to Cl(Ca) channels to cause a large I\(_{\text{Cl(Ca)}}\) probably in a nonlinear manner. Second, I\(_{\text{Cl(Ca)}}\) appears to be an increment detector of cytosolic Ca\(^{2+}\) because I\(_{\text{Cl(Ca)}}\) was found to correspond to the rate of rise of intracellular free Ca\(^{2+}\) rather than to its steady state levels of oocytes (Parker and Yao, 1994). The initial transient of I\(_{\text{Cl(Ca)}}\) might thus result from a large rate of rise of cytosolic Ca\(^{2+}\) at the beginning of Ca\(^{2+}\) influx.

To conclude, I\(_{\text{Cl(Ca)}}\) is a quantitatively unreliable measure of the underlying Ca\(^{2+}\) current as the relation between them is quite complex. Further detailed studies on I\(_{\text{Cl(Ca)}}\) channels (Hartzell, 1996) and diffusion-buffering processes will further increase the complexity. Therefore, direct measurement of ISOC in oocytes should greatly facilitate quantitative and molecular analysis of capacitative Ca\(^{2+}\) entry mechanisms.

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