

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 $I_{\text{Cl}(\text{Ca})}$ and unmasked a current in oocytes that was activated by InsP_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 $[\text{CaCl}_2]$ 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: $\text{Mg}^{2+} \ll \text{Ni}^{2+} \approx \text{Co}^{2+} \approx \text{Mn}^{2+} < \text{Cd}^{2+} \ll \text{Zn}^{2+} \ll \text{La}^{3+}$. It was also inhibited by niflumic acid, which is commonly used to block $I_{\text{Cl}(\text{Ca})}$. PMA partially inhibited the Ca^{2+} current, 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 $I_{\text{Cl}(\text{Ca})}$ 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_{CRAC} • I_{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_{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 inter-

nal store by InsP_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_{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 InsP_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 ($I_{\text{Cl}(\text{Ca})}$) (Barish, 1983; Miledi and Parker, 1984), with which one can monitor Ca^{2+} release and Ca^{2+} influx conveniently. Although $I_{\text{Cl}(\text{Ca})}$ remains a sensitive approach to

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¹Abbreviations used in this paper: BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; MeBAPTA, 1-(2-amino-5-methylphenoxy)-2-(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; NMDG, *N*-methyl-D-glucammonium; InsP_3 , inositol 1,4,5-trisphosphate; PMA, phorbol-12-myristate-13-acetate; NR, normal Ringer; I_{CRAC} , mammalian calcium release-activated calcium current; I_{SOC} , *Xenopus* stores-operated calcium current.

detect Ca^{2+} influx, a quantitative analysis of the 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 Ca^{2+} influx into oocytes, we explored approaches to record the calcium influx current directly. Injection of Ca^{2+} chelators was found to be a simple and efficient way to unmask the Ca^{2+} current by blocking the endogenous $I_{\text{Cl}(\text{Ca})}$, whereas blockers of anion currents interfered with the Ca^{2+} current. This Ca^{2+} current was characterized and shown to be similar in most but not all aspects to I_{CRAC} described in mast cells and lymphocytes (Hoth and Penner, 1992, 1993; Premack et al., 1994; Lewis and Cahalan, 1995). The store-operated Ca^{2+} current in *Xenopus* oocytes will be referred to as I_{SOC} to avoid implying that is exactly the same as the previously described I_{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 NaHCO_3 , 0.33 $\text{Ca}(\text{NO}_3)_2$, 0.41 CaCl_2 , 0.82 MgSO_4 , 5 HEPES, pH 7.4, supplemented with 50 $\mu\text{g}/\text{ml}$ 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 CaCl_2 , 1 MgCl_2 , 5 HEPES, titrated to pH 7.2 with NaOH. Ca^{2+} -free Ringer contained (in mM): 95 NaCl, 1 KCl, 5 MgCl_2 , 5 HEPES, titrated to pH 7.2 with NaOH. Iso-osmolar MgCl_2 medium (Mg70) was used to reduce leak current, which contained (in mM): 70 MgCl_2 , 10 HEPES, pH 7.2, titrated to pH 7.2 with NaOH. In experiments studying Ca^{2+} selectivity over monovalent cations, Ringers contained (in mM): 55 XCl, 30 CaCl_2 , 10 HEPES, X = Na, K, and *N*-methyl-D-glucamine (NMDG), titrated to pH 7.2 with NaOH, KOH, and HCl, respectively. Cl^- -free Ringer was prepared to examine the contribution of Cl^- to currents, which contained (in mM): 30 $\text{Ca}(\text{CH}_3\text{SO}_3)_2$, 55 KCH_3SO_3 , 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 Ca^{2+} , the Ringers had fixed concentration (in mM): 10 MgCl_2 and 10 HEPES-Na, pH 7.2, while CaCl_2 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 osmolarities.

Membrane currents were measured using a conventional two-electrode voltage clamp (Axoclamp-2B; Axon Instruments, Fos-

ter City, CA). Voltage and current electrodes were pulled and filled with 3 M KCl to have a resistance between 0.5–2 M Ω . Current output was lowpass-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 6.0.2 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 M Ω . Oocyte membrane potential was held at -60 mV. Intracellular injections were made using a pneumatic pressure ejection device (PV800; WPI, Inc., Sarasota, FL). All experiments were done at room temperature.

Thapsigargin, ionomycin, InsP_3 , 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_{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. Niflumic 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_{SOC} by Abolishing $I_{\text{Cl}(\text{Ca})}$ with MeBAPTA and EGTA

Most batches of oocytes did not have significant spontaneous Ca^{2+} influx. To induce Ca^{2+} influx, oocytes were incubated with 2 μM thapsigargin in Ca^{2+} -free Ringer for over 3 h (Petersen and Berridge, 1994). Alternatively, the Ca^{2+} influx was induced by InsP_3 , ionomycin and Ca^{2+} chelators (see below).

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, niflumic 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 CaCl_2 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 Cl^- equilibrium potential (Fig. 1 B, *b-a*). Currents were substantially reduced by 0.5 mM niflumic acid, and the I-V relation of the residual current in the presence of niflumic acid was still almost linear with a reversal potential of -20 mV (Fig. 1 C, *c-d*). This re-

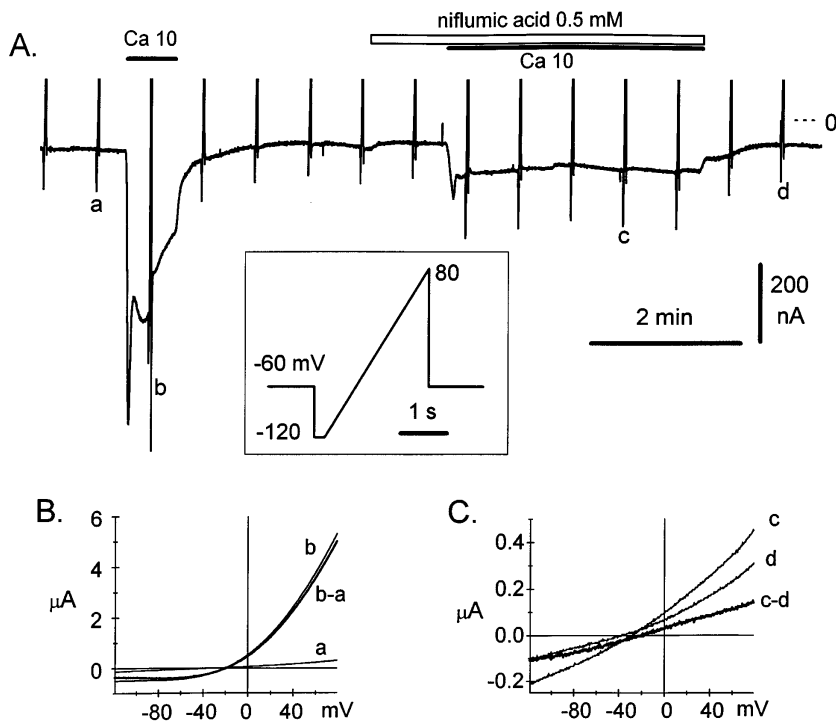


FIGURE 1. Effects of niflumic acid and I-V relation of niflumic acid-resistant current. (A) Oocytes treated with thapsigargin to induce long-term Ca²⁺ influx were voltage-clamped at -60 mV except for a brief voltage ramp from -120 to +80 mV (*inset*) applied every 40 s. Ca²⁺ entry was elicited by switching bath perfusion from Mg70 medium to NR supplemented with 10 mM CaCl₂ (*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.

sidual current in the presence of niflumic acid did not appear to be a Ca²⁺ current, which ought to be inwardly rectifying and to reverse at much more positive potentials.

A different approach using Ca²⁺ 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-μl oocyte volume. Most I_{Cl(Ca)} was blocked by both chelators within a minute after the injections. The reduced current was still mostly carried by Cl⁻ at that time because the I-V relation and reversal potential were close to that before the injection of the chelators (Figs. 2 *B* and 3 *B*). Currents induced by extracellular Ca²⁺ progressively decreased to less than one-thirtieth of the original peak of I_{Cl(Ca)} af-

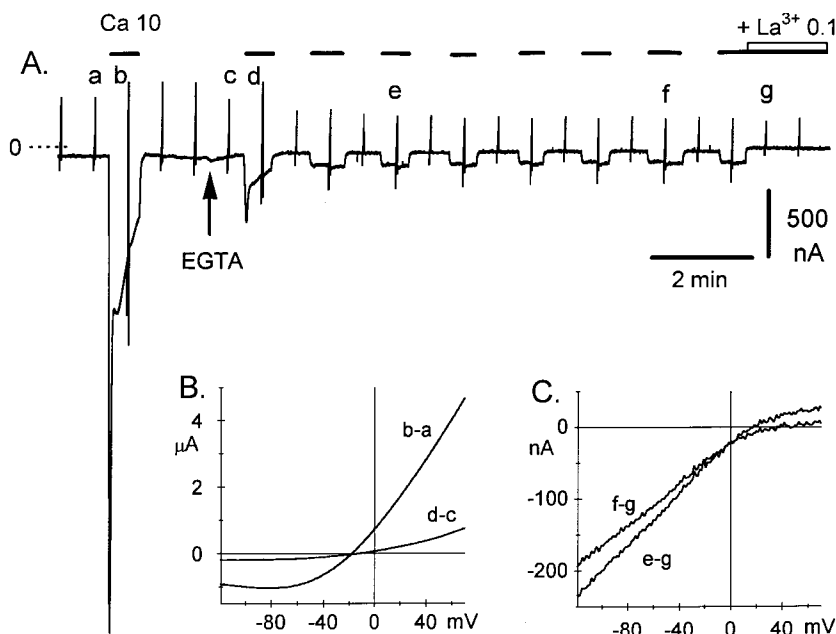


FIGURE 2. Direct recording of the Ca²⁺ current by injection of EGTA. Oocytes activated by thapsigargin were clamped at -60 mV and superfused with Mg70 medium. (A) The ramp-voltage shown in the inset of Fig. 1 was applied regularly at intervals of 40 s. CaCl₂ 10 mM in NR was applied as indicated (*solid bars*) to induce Ca²⁺ entry. Large outward I_{Cl(Ca)} traces responding to ramp voltage were truncated. 4 nmol EGTA was injected at time indicated (*arrow*). The Ca²⁺ current was blocked by 0.1 mM La³⁺ (*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 Ca²⁺ influx-induced current obtained before (*b-a*) and 1 min after (*d-c*) injection of EGTA. (C) I-V relation of Ca²⁺ influx-induced current obtained at ~4 (*e-g*) and 9 min (*f-g*) after the injection of EGTA.

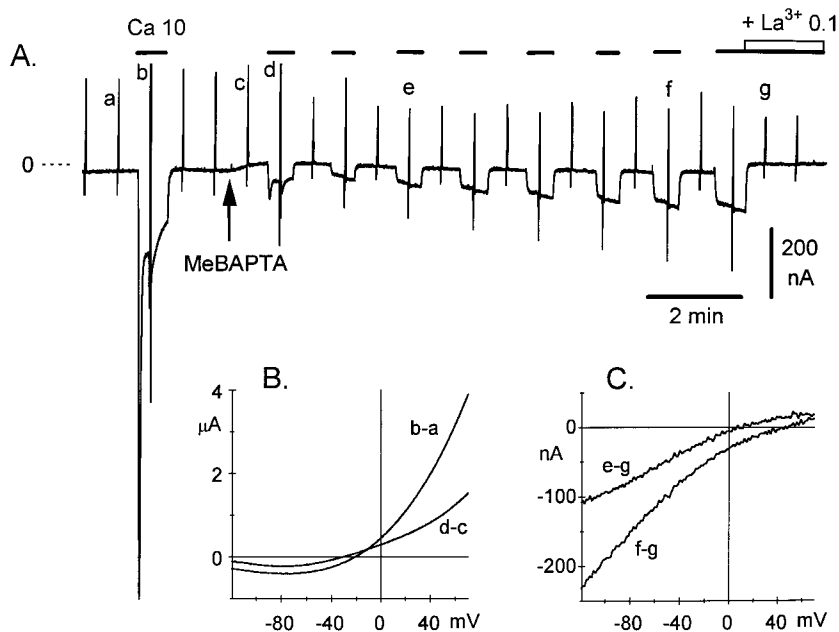


FIGURE 3. Direct recording of Ca^{2+} current by injection of MeBAPTA. Similar procedures were followed as described in legend of Fig. 2, except 4 nmol MeBAPTA was injected at time indicated (A, arrow). Ca^{2+} influx-induced currents b, d, e, and f were obtained at time as indicated in A. They were leak-subtracted and plotted versus membrane voltage in B and C.

ter two min post-injection. The transient phase of $I_{\text{Cl}(\text{Ca})}$ 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. I-V relations of the leak-subtracted currents obtained from EGTA- and MeBAPTA-loaded oocytes were plotted respectively (Figs. 2 C and 3 C). Typically, I-V curves of the Ca^{2+} influx-induced current changed drastically ~ 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 I-V 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 $I_{\text{Cl}(\text{Ca})}$ was gradually abolished and a Ca^{2+} current was finally revealed when Ca^{2+} buffers diffused throughout in the oocytes. Inhibition of $I_{\text{Cl}(\text{Ca})}$ was dose dependent on Ca^{2+} chelators. Injection of 0.4 nmol MeBAPTA per oocyte was sufficient to totally block transient $I_{\text{Cl}(\text{Ca})}$ 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 $I_{\text{Cl}(\text{Ca})}$. More than 1 nmol MeBAPTA and EGTA per oocyte was required to abolish $I_{\text{Cl}(\text{Ca})}$ completely (1 mM final assuming 1 μl oocyte volume).

MeBAPTA and EGTA showed some differences. The Ca^{2+} current increased by $140 \pm 20\%$ ($n = 5$) ~ 10 min after the injection of MeBAPTA (Fig. 3), whereas it decreased by $10 \pm 3\%$ ($n = 3$) in EGTA-injected oocytes during this period (Fig. 2). Although larger in amplitude, the I-V 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 M Ω ($n = 5$) 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 I-V relation. I_{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_{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 ~ 20 ms (Fig. 4 B). The transient current peak and plateau current had similar I-V relations (Fig. 4 C). Also the I-V relations obtained with the ramp-voltage command were close to those at steady state.

Dependence of Oocyte I_{SOC} on Extracellular Ca^{2+} Concentration

We then examined dependence of the Ca^{2+} current on extracellular Ca^{2+} concentrations in oocytes treated

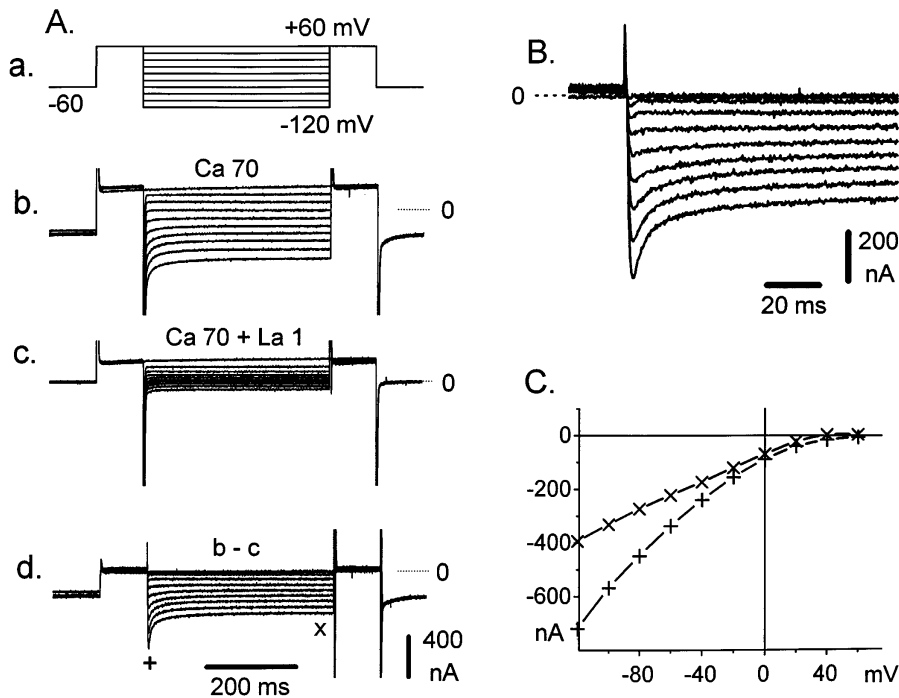


FIGURE 4. Transient I_{SOC} induced by membrane hyperpolarization. (A) Oocytes had been activated by thapsigargin and injected with EGTA. (a) The membrane voltage was stepped from the holding potential -60 mV to $+60$ mV for 100 ms, followed by hyperpolarizing steps in increments of -20 mV to -120 mV final. (b) Current was recorded in Ca70 medium containing (in mM) 70 $CaCl_2$, 10 HEPES, pH 7.2. (c) Leak current was acquired after addition of 1 mM $LaCl_3$ into Ca70 medium. (d) La^{3+} -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.

with thapsigargin and loaded with MeBAPTA. As the $CaCl_2$ concentration in perfusion Ringers was varied between 1 and 100 mM, the size of the Ca^{2+} current changed correspondingly (Fig. 5 A). The I-V relation of the current was measured at each concentration of $CaCl_2$ to ensure no $I_{Cl(Ca)}$ contamination occurred due to possible depletion of local Ca^{2+} buffers. Intervals between two $CaCl_2$ 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 $CaCl_2$ in oocytes loaded with MeBAPTA. Therefore application of $CaCl_2$ 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 $CaCl_2$ assuming the oocyte I_{SOC} pathway had no cooperative binding of Ca^{2+} . The best fit yielded an apparent activation constant $K_m = 11.5$ mM for $CaCl_2$ (Fig. 5 B). A similar value of $K_m = 10.9$ mM was obtained in a series of separate experiments, in which $CaCl_2$ 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, biionic approaches (Hess et al., 1986) could not be applied to study the ion selectivity of I_{SOC} . In addition, exact mea-

surement 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 (I_c) was evident in oocytes in Ca^{2+} -free Ringer and was inhibited by both extracellular Ca^{2+} and Mg^{2+} (Arellano et al., 1995). Thus Ca^{2+} -free Ringer was replaced by Mg70 medium to reduce the leak current induced by removal of Ca^{2+} . Oocytes appeared healthy for at least 20 h in this saline lacking both monovalent cations and Ca^{2+} . 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 Ca^{2+} , 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, La^{3+} was used to block I_{SOC} . The current acquired after adding La^{3+} was then taken as the leak current for subtraction. La^{3+} is by no means a selective antagonist of I_{SOC} . Like another lanthanide ion, Gd^{3+} (Arellano et al., 1995), it could additionally inhibit I_c . The La^{3+} -sensitive difference current might thus contain residual I_c that was not blocked by Ca^{2+} . In this case, the current would reverse at a potential less positive than the pure I_{SOC} .

To examine the selectivity of I_{SOC} channels for Ca^{2+} over Na^+ and K^+ , all extracellular monovalent cations were replaced alternately by pure Na^+ , K^+ , or NMDG.

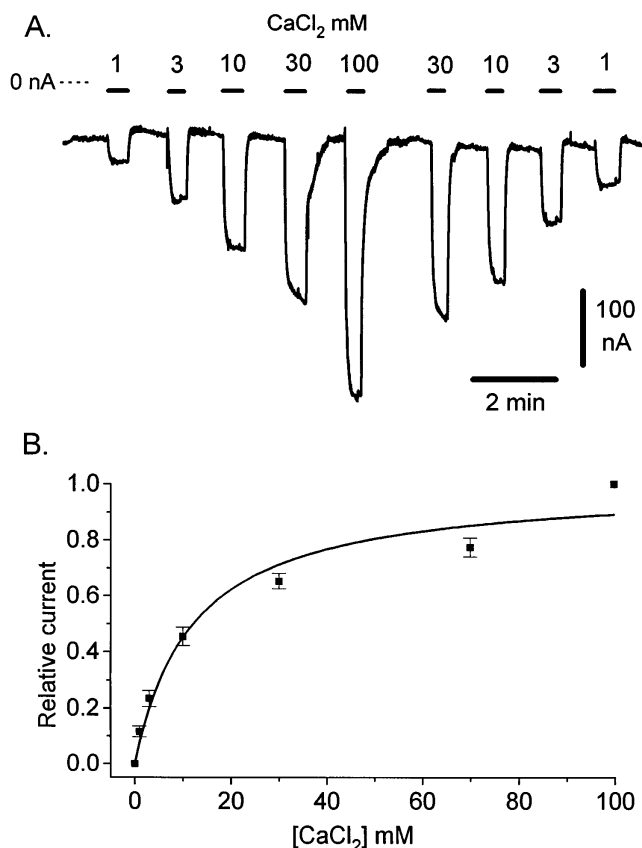


FIGURE 5. Dependence of oocyte I_{SOC} on extracellular concentration of Ca^{2+} . Ca^{2+} entry in oocytes was activated by thapsigargin. The oocyte was then injected with MeBAPTA and superfused with Mg70 medium while clamping at -60 mV. (A) The Ca^{2+} current was elicited by perfusion of Ringers containing 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 symbols and error bars represent means \pm SE ($n = 6$). The smooth curve is the best fit of the equation $I/I_{\max} = 1/(1 + K_m/[CaCl_2])$ where I is the current amplitude, I_{\max} the saturating value of I at infinite $[CaCl_2]$, and K_m is the Michaelis-Menten activation constant.

Also, the contribution of Cl^- was assessed by replacement with $CH_3SO_3^-$. Recordings were made in thapsigargin-treated and MeBAPTA-loaded oocytes. Currents induced in the above solutions had almost similar amplitudes at a holding potential of -60 mV (Fig. 6 A), indicating that Na^+ , K^+ , and Cl^- ions did not contribute to the current significantly. Further, I-V relations were obtained in the above solutions (Fig. 6 B). No significant differences in I-V relation were observed when Na^+ , K^+ , and Cl^- were removed totally from the extracellular medium, indicating that the Ca^{2+} current was highly selective for Ca^{2+} over Na^+ and K^+ , and Cl^- did not affect the Ca^{2+} current.

Ca^{2+} was then replaced by Ba^{2+} and Sr^{2+} to examine their permeability through this oocyte I_{SOC} pathway.

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 I_{SOC} by Metal Ions and Niflumic Acid

Whereas Sr^{2+} and Ba^{2+} permeated this oocyte I_{SOC} pathway readily, some transition metal ions, Ni^{2+} , Co^{2+} , Mn^{2+} , Cd^{2+} , and La^+ 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 I_{SOC} was reduced by $24 \pm 4\%$ ($n = 6$), $26 \pm 4\%$ ($n = 6$), $27 \pm 2\%$ ($n = 6$), $65 \pm 3\%$ ($n = 6$) of the control by 1 mM Ni^{2+} , Co^{2+} , Mn^{2+} , or Cd^{2+} , respectively. Zn^{2+} and La^{3+} blocked I_{SOC} completely at 1 mM, and concentrations for half-inhibition (IC_{50}) were ~ 40 μ M for Zn^{2+} ($n = 4$) and 0.3 μ M for La^{3+} ($n = 3$), respectively. In experiments evaluating the inhibitory effect of Mg^{2+} , I_{SOC} 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 (Mg70 medium plus 10 mM $CaCl_2$). Mg^{2+} had a very weak inhibitory effect on I_{SOC} , inhibiting by only $24 \pm 4\%$ at 70 mM $MgCl_2$ ($n = 5$).

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

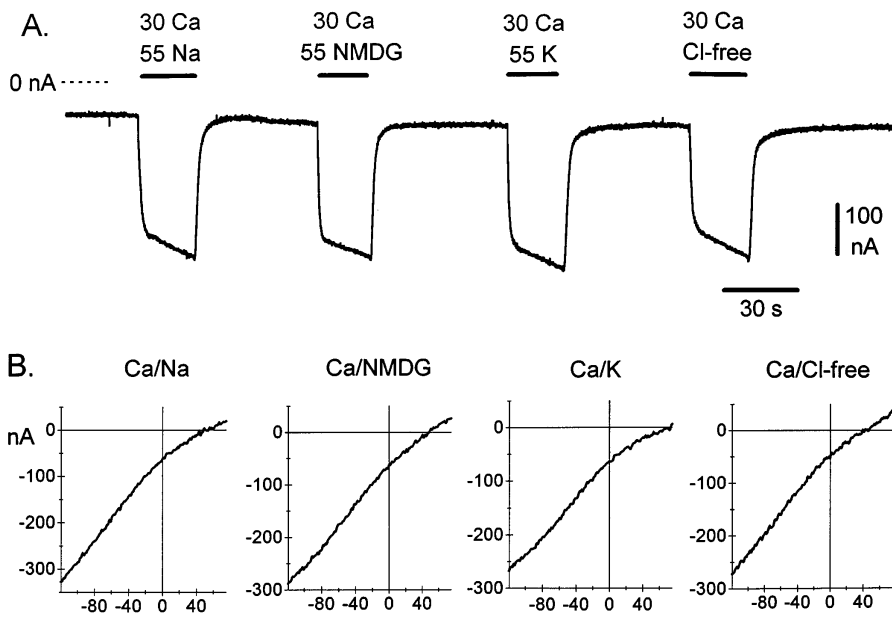


FIGURE 6. Selectivity of I_{SOC} for Ca^{2+} over K^{+} and Na^{+} . (A) Oocytes activated by thapsigargin and pre-loaded with MeBAPTA were clamped at -60 mV and perfused with Mg70 medium. Introduction of Ca^{2+} -containing solutions into bath was indicated as solid bars in the figure. The Ca^{2+} current was induced subsequently by solutions containing (in mM): 30 $CaCl_2$, 55 XCl, and 10 HEPES, pH 7.2, where X = Na, NMDG, or K, and then by a Cl^{-} -free solution containing (in mM): 30 $Ca(CH_3SO_3)_2$, 55 KCH_3SO_3 , and 10 HEPES, pH 7.2. (B) I-V relations were acquired in the above solutions using the same ramp-voltage protocol as in the inset of Fig. 1. Leak current was obtained after adding $30 \mu M La^{3+}$ to the above corresponding solutions to block the Ca^{2+} current.

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. 9 B).

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+}

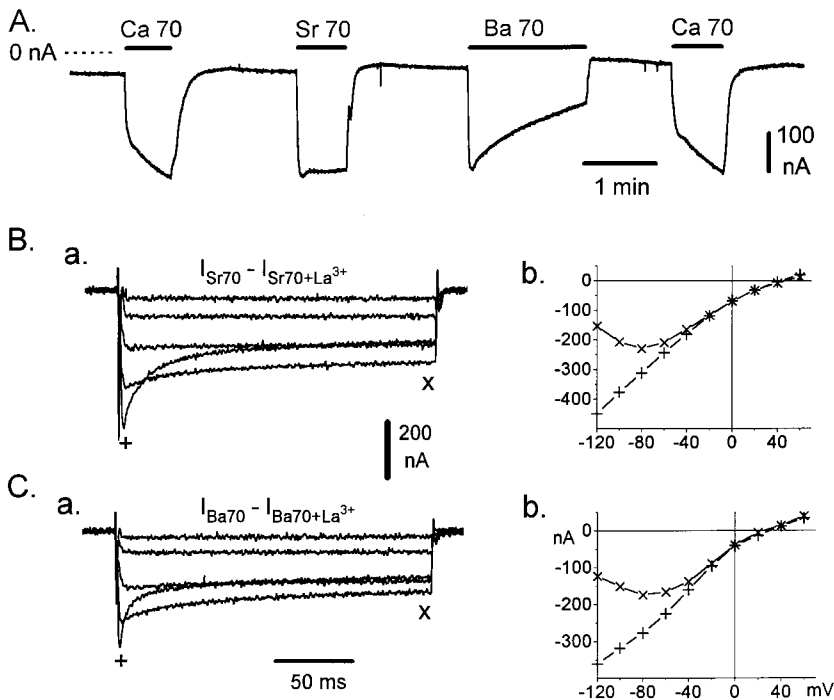


FIGURE 7. Conductances for Sr^{2+} and Ba^{2+} in the I_{SOC} pathway. (A) Oocytes activated by thapsigargin and preloaded with MeBAPTA were clamped at -60 mV and perfused with Mg70 medium. Current was induced by perfusing oocytes successively with Ca70, Sr70, and Ba70, containing (in mM): 70 MCl_2 , 10 HEPES-Na, pH 7.2, where M = Ca, Sr, and Ba, respectively. (B and C) Sr^{2+} and Ba^{2+} currents were recorded applying a pulse-voltage protocol similar to that shown in Fig. 4. A a. Sr^{2+} current traces (B a) and Ba^{2+} current traces (C a) were elicited with voltage steps respectively to $+40$, 0 , -40 , -80 , and -120 mV and have been corrected for leak currents measured after adding $3 \mu M La^{3+}$ to block Sr^{2+} and Ba^{2+} currents. I-V relations of initial transient current (+) and sustained current (x) were plotted respectively for Sr^{2+} (B b) and Ba^{2+} currents (C b).

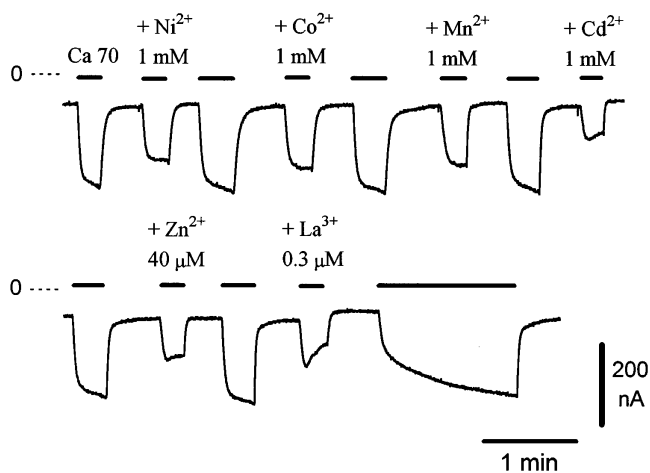


FIGURE 8. Inhibition of oocyte I_{SOC} by metal ions. Oocytes treated with thapsigargin to induce Ca^{2+} influx and injected with MeBAPTA were held at -60 mV, superfused with Mg70 medium and repetitively challenged with 10 mM $CaCl_2$ in NR (solid bars) alone (first bar and subsequent unlabeled bars) or in combination with various metal ions at the indicated concentrations.

influx was induced by 10 mM Ca^{2+} before the injection of $InsP_3$. About 2 min after the injection of $InsP_3$, Ca^{2+} influx was clearly activated as indicated by a large $I_{Cl(Ca)}$ when bath solution was switched from Ca^{2+} -free Ringer to NR with Ca^{2+} concentration added to 10 mM. EGTA was subsequently injected to block $I_{Cl(Ca)}$. About 1 min after the injection, Ca^{2+} influx-induced $I_{Cl(Ca)}$ 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 $InsP_3$ as the two injection pipettes were close to each other. The Ca^{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^{2+} current activation by $InsP_3$ in oocytes pre-injected with MeBAPTA is depicted in Fig. 10 C.

Ionomycin induced Ca^{2+} influx quickly. Ca^{2+} influx was not significant before bath application of ionomycin as monitored by switching from Ca^{2+} -free Ringer to NR with 10 mM Ca^{2+} . Ionomycin induced Ca^{2+} release as indicated by $I_{Cl(Ca)}$ of several μA in Ca^{2+} -free Ringer (Fig. 11 A). A sustained Ca^{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^{2+} current was obtained after injection of EGTA (Fig. 11 B, *a-b*). Full activation of the Ca^{2+} current in oocytes pre-injected with MeBAPTA took only several min (Fig. 11 C). To ascertain that the ionomycin-induced Ca^{2+} current was not due to Ca^{2+} transport by residual ionomycin, the oocytes treated with thapsigargin were exposed to ionomycin for 3 min to examine whether ionomycin could induce an additional

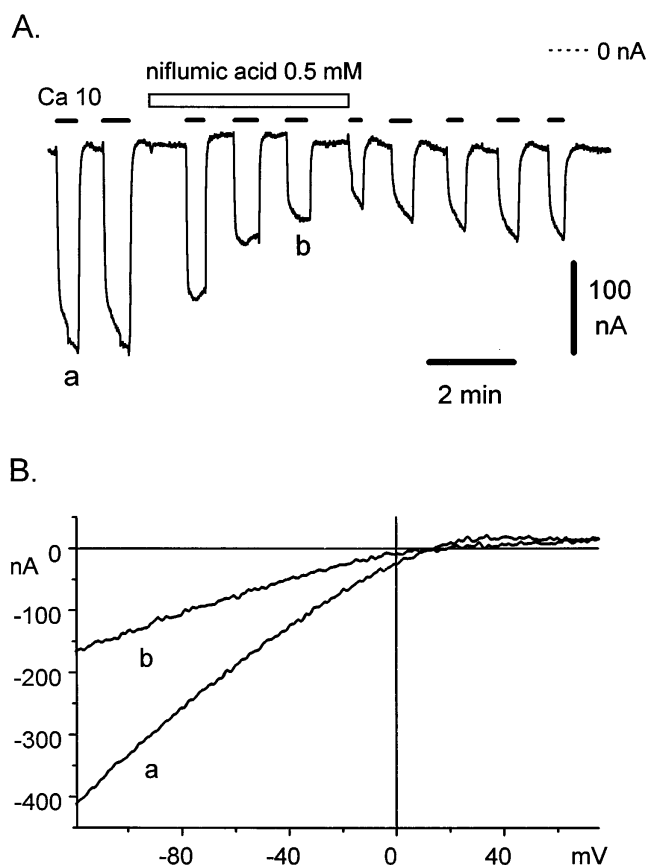


FIGURE 9. Inhibitory effect of niflumic acid on I_{SOC} . Oocytes treated with thapsigargin and injected with MeBAPTA were held at -60 mV. (A) I_{SOC} was repetitively elicited by 10 mM $CaCl_2$ in NR (solid bars). Bath application of niflumic acid 0.5 mM was indicated with a hollow bar. (B) I-V relations of the leak-subtracted current before (*a*) and during (*b*) the application of niflumic acid. These were measured at times indicated in A, in which for simplicity the ramp responses are not shown.

Ca^{2+} current. No significant change of Ca^{2+} current was measured after incubation of 2 μM ionomycin ($n = 4$), indicating that ionomycin is an electroneutral carrier and does not mediate significant Ca^{2+} current by itself. The concentration dependence of the Ca^{2+} current induced by ionomycin on extracellular Ca^{2+} and the inhibitory potency of the metal ions were identical to those measured in thapsigargin-treated oocytes ($n = 3$), which further indicated that the ionomycin-induced Ca^{2+} current was not carried by ionomycin and was activated at a step downstream to depletion of Ca^{2+} store.

The time course of the I_{SOC} activation by EGTA was slow and the current size quite variable (Fig. 12). Data acquired from oocytes from three different donors were pooled together to characterize the variability in activation of I_{SOC} by EGTA. Oocyte I_{SOC} appeared to be fully activated in 3 h after the injection of 3 – 5 nmol EGTA although the final size of the Ca^{2+} current varied severalfold in oocytes from different frogs (Fig. 12).

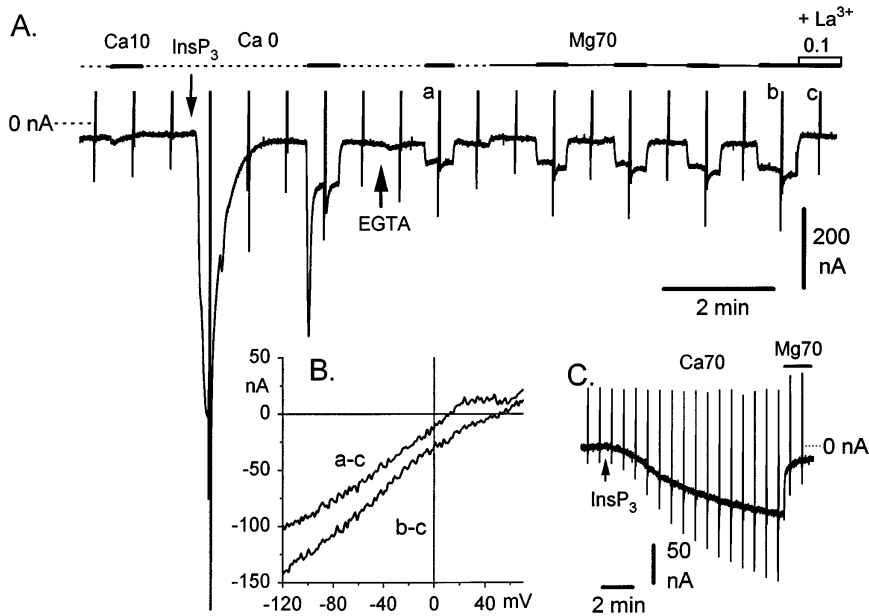


FIGURE 10. Activation of I_{SOC} by $InsP_3$. (A) The oocyte was superfused with Ca^{2+} -free Ringer (dotted line) at the beginning, then with Mg70 medium (thin solid line) 7 min later. Ca^{2+} influx was elicited by 10 mM $CaCl_2$ in NR (thick bars). The ramp-voltage command was applied periodically to obtain the I-V relation. $InsP_3$ 32 pmol (first arrow) was injected to induce Ca^{2+} release and influx. EGTA 8 nmol (second arrow) was then injected to block $I_{Cl(Ca)}$. (B) I-V relation of the immediate and later Ca^{2+} currents (a-c and b-c) after subtraction of the leak current (c) obtained after adding La^{3+} 0.1 mM (A, hollow bar). (C) Time course of I_{SOC} activation by injection of 120 pmol $InsP_3$ (arrows). The oocyte was preinjected with MeBAPTA and recorded in Ca70 medium. Vertical current traces were evoked by the standard ramp-voltage command.

The leak conductance did not vary systematically during this period (Fig. 12). Little or no Ca^{2+} 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 Ca^{2+} currents induced by $InsP_3$, ionomycin and EGTA. No marked difference in ion selectivity could be detected among the Ca^{2+} currents activated by these different means. No obvious I_{SOC} was found in oocytes incubated for 20 h in Ca^{2+} -free Ringer supplemented with 0.1 mM EGTA ($n = 3$), although the transient Ca^{2+} -influx-dependent $I_{Cl(Ca)}$ described by Petersen and

Berridge (1994) was seen. When the Ca^{2+} -free Ringer was temporarily replaced with normal oocyte Ringer, resting potentials were around -50 mV and input resistance was $1 M\Omega$.

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 Ca^{2+} 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 Ca^{2+} influx in oo-

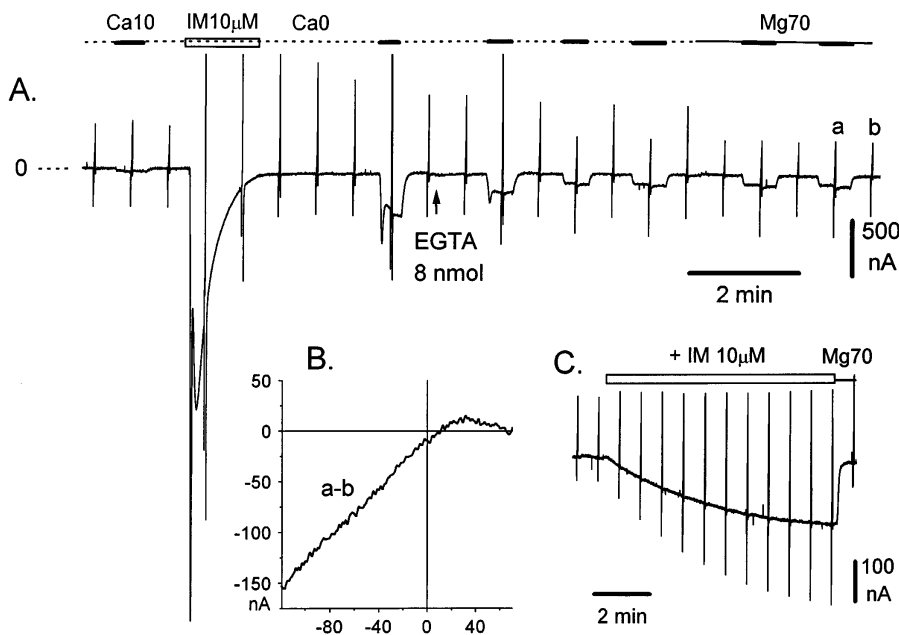


FIGURE 11. Activation of I_{SOC} by ionomycin. (A) Oocyte was clamped at -60 mV and perfused with Ca^{2+} -free Ringer (dotted line) or Mg70 medium (thin solid line). Ca^{2+} influx activity was tested by switching bath to $CaCl_2$ 10 mM in NR (thick bar). Ionomycin $10 \mu M$ in Ca^{2+} -free Ringer supplemented with 1 mM EGTA (hollow bar) was superfused to induce Ca^{2+} release. EGTA 8 nmol was injected at the time indicated (arrow). (B) I-V relation of the Ca^{2+} current (a-b) after $I_{Cl(Ca)}$ was abolished by EGTA. The leak current (b) was obtained in Mg70 medium. (C) The time course of I_{SOC} activation by ionomycin. This oocyte was preloaded with MeBAPTA and recorded in Ca70 medium. Vertical current traces were elicited by the ramp-voltage command.

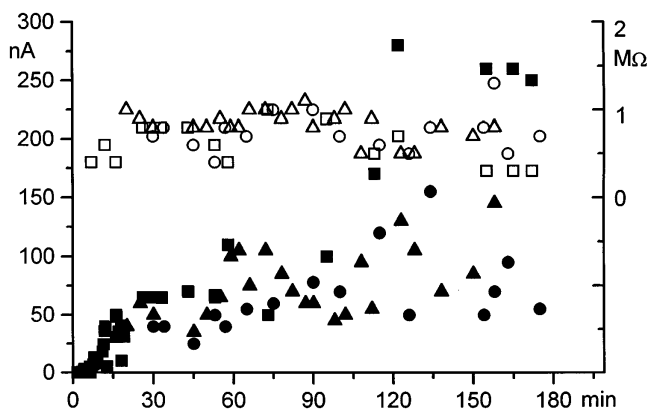


FIGURE 12. Time course of oocyte I_{SOC} activation by EGTA. Oocytes were injected with ~ 4 nmol EGTA. I_{SOC} was measured in 70 mM $CaCl_2$ at -60 mV. I_{SOC} was continuously recorded for the first 20 min after the injection of EGTA ($n = 5$). Each subsequent solid symbol represents I_{SOC} measured from a single oocyte and calibrated by the left-hand ordinate axis, whereas each open symbol gives the corresponding input resistance referred to the right-hand axis. Squares, circles, and triangles indicate oocytes obtained from three animals.

cytes, Ca^{2+} current was measured before and after bath application of the phorbol ester PMA. The only effect of PMA on I_{SOC} was inhibitory in oocytes activated by ionomycin ($n = 9$) or thapsigargin ($n = 19$). I_{SOC} declined monotonically with time during perfusion of PMA. The inhibitory rate increased with concentration of PMA. Typically, I_{SOC} was reduced by $49.7 \pm 3.4\%$ ($n = 8$) 6 min after perfusion of PMA $1 \mu M$ (Fig. 13 A). The leak conductance was simultaneously decreased but by an unknown mechanism. Calphostin C was used to confirm that 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 $2 \mu M$ and kept under room fluorescent light for more than 0.5 h before recording started, as recommended by Bruns et al. (1991). I_{SOC} was reduced by only $14.4 \pm 2.9\%$ ($n = 5$) 6 min after bath application of PMA $1 \mu M$ in the calphostin C-injected oocytes, indicating that inhibitory action of PMA was mostly blocked. I_{SOC} was 93 ± 15 nA ($n = 5$) and 104 ± 14 nA ($n = 4$) in calphostin C-injected and control oocytes, respectively, suggesting that I_{SOC} was not significantly modulated by protein kinase C at the resting state.

DISCUSSION

This study describes I_{SOC} in *Xenopus* oocytes using a conventional two-electrode voltage-clamp technique. This method preserves the oocyte cytosol during prolonged recording, in contrast to the whole-cell patch-clamp technique used in other studies (Hoth and Penner, 1992, 1993; Premack et al., 1994; Lewis and Cahalan, 1995). I_{SOC} was isolated by blocking native $I_{Cl(Ca)}$

with microinjected Ca^{2+} chelators (Figs. 2 and 3). Compared with I_{CRAC} described formerly in mast cells and Jurkat lymphocytes (Hoth and Penner, 1992, 1993; Premack et al., 1994; Lewis and Cahalan, 1995), oocyte I_{SOC} had a similar inwardly rectifying I-V relation (Figs. 2–4), high Ca^{2+} selectivity over Na^+ and K^+ (Fig. 6), and sequence of inhibitory potency by other ions, $Mg^{2+} \ll Ni^{2+} \approx Co^{2+} \approx Mn^{2+} < Cd^{2+} \ll Zn^{2+} \ll La^{3+}$ (\ll represents about one order of magnitude difference or more) (Fig. 8). The oocyte I_{SOC} pathway had similar instantaneous conductances for Ca^{2+} , Sr^{2+} , and Ba^{2+} , yet Ba^{2+} and Sr^{2+} currents appeared to inactivate more strongly than Ca^{2+} current (Fig. 7). By contrast the I_{CRAC} pathway conducts Ca^{2+} about twice as well as Ba^{2+} and Sr^{2+} (Lewis and Cahalan, 1995). In addition, the dependence of I_{SOC} on extracellular $CaCl_2$ concentration had an apparent $K_m = 11.5$ mM in the oocytes (Fig. 5), higher than that of I_{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_{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^{2+} from internal stores of oocytes. Total I_{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 $1 pA pF^{-1}$ 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_{SOC} should be possible on a giant membrane patch with size of around 30 μm (Hilgemann, 1995). This may offer the opportunity to separate the plasma membrane I_{SOC} pathway from the internal Ca^{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 $InsP_3$ 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 $I_{Cl(Ca)}$

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

stimulated by serotonin. Similar properties of niflumic acid-resistant current were observed in this whole-cell study. This current reversed at about -20 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 $I_{\text{Cl}(\text{Ca})}$ because this drug also inhibited oocyte I_{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_{CRAC} in rat basophilic leukemia 2H3 cells (Reinsprecht et al., 1995).

The calcium chelators block $I_{\text{Cl}(\text{Ca})}$ 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 $\text{Cl}(\text{Ca})$ 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_{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_{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 $I_{\text{Cl}(\text{Ca})}$ in oocytes. This might arise from the smaller unitary conductance of CRAC or SOC channels and possibly larger distance between $\text{Cl}(\text{Ca})$ channels and SOC channels in oocytes. I_{SOC} was found to be consistently larger in oocytes injected with MeBAPTA than that with EGTA. Also, in oocytes buffered with MeBAPTA, I_{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_{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_{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_{SOC} was needed. EGTA would then be the better choice in this case, because neither I_{SOC} nor the membrane leak was significantly affected by this chelator.

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

$I_{\text{Cl}(\text{Ca})}$ 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 $I_{\text{Cl}(\text{Ca})}$ is more than 10 times larger than the underlying calcium influx current (see Figs. 2 and 3). However, the relation between $I_{\text{Cl}(\text{Ca})}$ and I_{SOC} is not simple. Several discrepancies are obvious. First, $I_{\text{Cl}(\text{Ca})}$ evoked by Ca^{2+} influx has an initially large transient

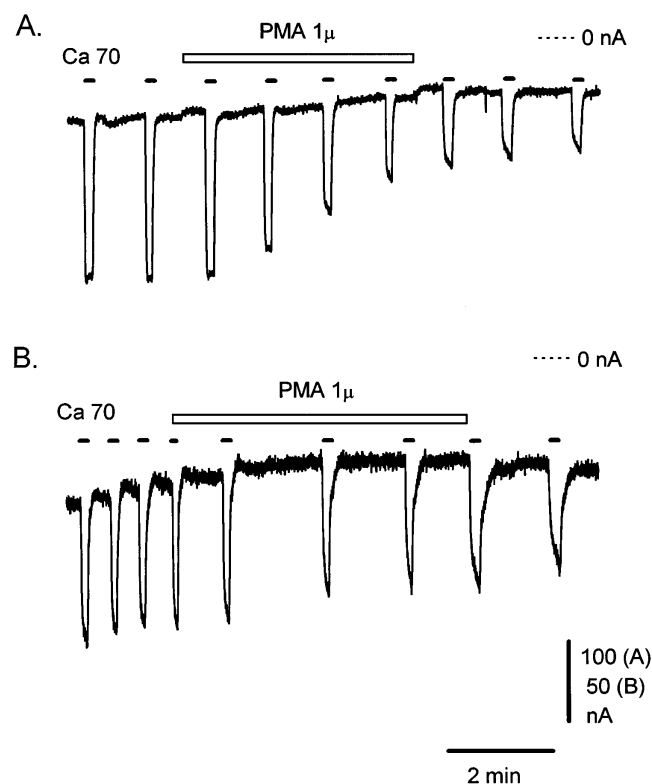


FIGURE 13. Inhibition of I_{SOC} by activation of protein kinase C. Oocytes were treated with ionomycin and injected with EGTA. Ca^{2+} current was induced by switching bath perfusion from Mg70 medium to Ca70 medium repetitively as indicated by the solid bars. Bath application of PMA $1 \mu\text{M}$ (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 $I_{\text{Cl}(\text{Ca})}$ 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 $I_{\text{Cl}(\text{Ca})}$ inactivates and recovers in about one minute. Although different mechanisms were proposed, approximately similar characteristics of the $I_{\text{Cl}(\text{Ca})}$ 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 $I_{\text{Cl}(\text{Ca})}$, T_{in} (Parker et al., 1985). T_{in} appeared to rise and decay more rapidly than the transient $I_{\text{Cl}(\text{Ca})}$ in thapsigargin- and the ionophore-treated oocytes. In addition, T_{in} appeared to have a different onset shape. Simultaneous recording of $I_{\text{Cl}(\text{Ca})}$ and Ca^{2+} fluorescence showed that T_{in} reflects InsP_3 -dependent Ca^{2+} -induced Ca^{2+} release (Yao and Parker, 1993). Consistent with this, the hump component of the $I_{\text{Cl}(\text{Ca})}$ 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 $I_{\text{Cl}(\text{Ca})}$ does not require Ca^{2+} influx via the I_{SOC} pathway per se. The InsP_3 -dependent Ca^{2+} -induced Ca^{2+} release mechanism, however, fails to explain the transient $I_{\text{Cl}(\text{Ca})}$ 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 $I_{\text{Cl}(\text{Ca})}$ 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 I_{SOC} in this study. Besides, injection of as little as 120 pmol slow buffer EGTA (final 120 μM assuming 1 μl oocyte volume) was sufficient to totally block the transient $I_{\text{Cl}(\text{Ca})}$ 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 $I_{\text{Cl}(\text{Ca})}$, rather than between stores depletion and Ca^{2+} influx.

Alternatives to regenerativity to interpret the nonlinearity of Ca^{2+} influx-induced $I_{\text{Cl}(\text{Ca})}$ should be considered. First, diffusion and buffering processes could contribute to the nonlinearity. Local free $[\text{Ca}^{2+}]$ 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 $[\text{Ca}^{2+}]$. 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 $\text{Cl}(\text{Ca})$ channels and hence no or small $I_{\text{Cl}(\text{Ca})}$. With large influx of Ca^{2+} , the Ca^{2+} buffers would be depleted locally, so that more free Ca^{2+} would spread to $\text{Cl}(\text{Ca})$ channels to cause a large $I_{\text{Cl}(\text{Ca})}$, probably in a nonlinear manner. Second, $I_{\text{Cl}(\text{Ca})}$ appears to be an increment detector of cytosolic Ca^{2+} because $I_{\text{Cl}(\text{Ca})}$ 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 $I_{\text{Cl}(\text{Ca})}$ might thus result from a large rate of rise of cytosolic Ca^{2+} at the beginning of Ca^{2+} influx.

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

We would like to thank Dr. Ian Parker and Dr. Pierre Vincent for their critical discussion and comments on the manuscript. We appreciate Dr. Stephen Adams for providing MeBAPTA.

This study was supported by grant RG520/95 from the Human Frontier Science Program and by the Howard Hughes Medical Institute.

Original version received 12 February 1997 and accepted version received 10 April 1997.

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