Am J Physiol Cell Physiol
278: C667–C675, 2000.

Ca^{2+}-dependent activation of Cl^- currents in *Xenopus* oocytes is modulated by voltage

NICK CALLAMARAS AND IAN PARKER
Laboratory of Cellular and Molecular Neurobiology, Department of Neurobiology and Behavior, University of California Irvine, Irvine, California 92697-4550

Callamaras, Nick, and Ian Parker. Ca^{2+}-dependent activation of Cl^- currents in *Xenopus* oocytes is modulated by voltage. *Am J Physiol Cell Physiol* 278: C667–C675, 2000.—Ca^{2+}-activated Cl^- currents ($I_{Cl,Ca}$) were examined using fluorescence confocal microscopy to monitor intracellular Ca^{2+} liberation evoked by flash photolysis of caged inositol 1,4,5-trisphosphate ($InsP_3$) in voltage-clamped *Xenopus* oocytes. Currents at +40 mV exhibited a steep dependence on $InsP_3$ concentration ([$InsP_3$]), whereas currents at −140 mV exhibited a higher threshold and more graded relationship with [$InsP_3$]. Ca^{2+} levels required to half-maximally activate $I_{Cl,Ca}$ were about 50% larger at −140 mV than at +40 mV, and currents evoked by small Ca^{2+} elevations were reduced >25-fold. The half-decay time of Ca^{2+} signals shortened at increasingly positive potentials, whereas the decay of $I_{Cl,Ca}$ lengthened. The steady-state current-voltage (*I-V*) relationship for $I_{Cl,Ca}$ exhibited outward rectification with weak photolysis flashes but became more linear with stronger stimuli. Instantaneous *I-V* relationships were linear with both strong and weak stimuli. Current relaxations following voltage steps during activation of $I_{Cl,Ca}$ decayed with half-times that shortened from about 100 ms at +10 mV to 20 ms at −160 mV. We conclude that $InsP_3$-mediated Ca^{2+} liberation activates a single population of Cl^- channels, which exhibit voltage-dependent Ca^{2+} activation and voltage-independent instantaneous conductance.

calcium-activated chloride current; voltage dependence; inositol 1,4,5-trisphosphate

NUMEROUS Ca^{2+}-activated Cl^- channels are present in the plasma membrane of *Xenopus* oocytes (13) and serve to generate the fertilization potential that provides a fast electrical block to polyspermy in the egg (7). Voltage-clamp recordings of Cl^- current provide a convenient reporter of subplasmalemmal Ca^{2+} concentration ([Ca^{2+}]) and are widely used to study both endogenous Ca^{2+} signaling pathways in the oocyte and to monitor the expression of exogenous Ca^{2+}-mobilizing receptors (8, 14). Ca^{2+}-activated Cl^- currents ($I_{Cl,Ca}$), however, show complex time- and dose-dependent characteristics that complicate interpretation of the underlying Ca^{2+} signals (11, 21). In particular, it remains unclear whether they are generated through a single population of Cl^- channels or through two or more channel types that have different sensitivities to Ca^{2+} (3, 11).

This problem is particularly vexing in the case of responses mediated by the inositol 1,4,5-trisphosphate ($InsP_3$) messenger pathway. Ca^{2+} signals evoked by $InsP_3$ involve a rapid, transient liberation of Ca^{2+} from endoplasmic reticulum stores, followed by influx of extracellular Ca^{2+} via a plasma membrane pathway activated by store depletion (2, 23). These two sources of Ca^{2+} can be discriminated by recording $I_{Cl,Ca}$ evoked by hyperpolarizing voltage steps because entry of extracellular Ca^{2+} depends on the electrochemical gradient across the cell membrane, whereas intracellular Ca^{2+} mobilization is largely independent of membrane potential (11, 19, 28). We had previously interpreted the resulting Cl^- currents as arising through a single class of Cl^- channels (28), but a more detailed electrophysiological analysis led Hartzell (11) to propose the involvement of two channel types: one generating an outwardly rectifying current activated by both intracellular Ca^{2+} liberation and Ca^{2+} influx and a second channel with lower affinity for Ca^{2+} that is activated selectively by the high subplasmalemmal Ca^{2+} levels resulting from Ca^{2+} influx during hyperpolarizing voltage steps. An alternative hypothesis, however, is that only a single class of $I_{Cl,Ca}$ channels is present in the oocyte, but that the sensitivity of these channels for Ca^{2+} is voltage dependent (12).

To study the influence of membrane potential on the Ca^{2+} sensitivity of Cl^- channels, we recorded Cl^- currents and cytosolic Ca^{2+} signals generated by Ca^{2+} arising from transient liberation of Ca^{2+} from intracellular stores induced by flash photolysis of caged $InsP_3$. Currents recorded at positive holding potentials exhibited a lower threshold and steeper dose dependence on $InsP_3$ concentration ([$InsP_3$]) than corresponding currents at hyperpolarized potentials, even though intracellular Ca^{2+} signals monitored by confocal microfluorimetry were similar at both voltages. Furthermore, the steady-state current-voltage (*I-V*) relationship of the Cl^- current changed progressively from strongly outward rectifying to linear with increasing intracellular [Ca^{2+}]. These results are consistent with the presence of a single class of Cl^- channels that show increasing sensitivity to Ca^{2+} at more positive potentials.

METHODS

Immature (stage V and VI) oocytes of *Xenopus laevis* were obtained as previously described (25). Frogs were anesthe-

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

 0363-6143/00 $5.00 Copyright © 2000 the American Physiological Society

tized by immersion in a 0.17% aqueous solution of MS-222 (3-aminobenzoic acid ethyl ester) for 15 min, and a small portion of the ovary was removed surgically through an abdominal incision, after which the wound was sutured and animals were allowed to recover. After manual removal of epithelial layers, oocytes were loaded 30–60 min before recording with caged InsP_3 [*myo*-inositol 1,4,5-trisphosphate, *P*4(5)-1-(2-nitrophenyl) ethyl ester] together with the low-affinity Ca^{2+} indicator Oregon Green 488 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-5N (OG-5N) to final intracellular concentrations of about 5 and 50 μM, respectively (26).

Recordings were made at room temperature, with oocytes bathed in normal Ringer solution (in mM, 120 NaCl, 2 KCl, 1.8 $CaCl_2$, 5 HEPES, at pH about 7.2). Ca^{2+}-free solution contained no added Ca^{2+}, and additionally 1 mM EGTA and 5 mM Mg^{2+}. Measurements of membrane currents were obtained using a two-electrode voltage clamp (GeneClamp 500; Axon Instruments; Foster City, CA), with KCl-filled microelectrodes broken to resistances of 1–3 MΩ. Simultaneously, measurements were made of intracellular Ca^{2+} signals (monitored by OG-5N fluorescence) evoked by photostimulation, using a linescan confocal microscope and flash photolysis system as described previously (4, 17). In brief, a custom-built confocal scanner assembly was interfaced to an Olympus IX 70 inverted microscope fitted with a ×40 oil-immersion objective (NA 1.35). Fluorescence excited in the oocyte by a diffraction-limited spot of 488 nm light from an argon ion laser was monitored through a confocal aperture at $\lambda > 510$ nm while the spot was repeatedly scanned along a 50-μm line. The microscope was focused at the depth of the pigment granules within the oocyte, which lie about 2 μm inward from the cell surface. Measurements are presented as a ratio relative to the resting fluorescence before stimulation (ΔF/F) and represent an average across the scan line. Scans were obtained at a rate of 125 Hz, and current records were low-pass filtered at 50 Hz. The low-affinity indicator OG-5N was used to avoid saturation during strong InsP_3-evoked Ca^{2+} signals and provide a fluorescence signal linearly proportional to $[Ca^{2+}]$ over the range of interest. The dissociation constant of OG-5N is about 31 μM [measured using Molecular Probes buffer kit 3 in the presence of 1 mM Mg^{2+} (24)], and we observed a maximal fluorescence change of 12.6 following injections of saturating amounts of Ca^{2+} into oocytes ($n = 3$) through a micropipette filled with 100 mM $CaCl_2$. Thus for small Ca^{2+} signals, the dye provides a fluorescence change of ~0.20 ΔF/F per micromole of free Ca^{2+}.

Flashes of ultraviolet (UV; 340–400 nm) light, derived from a continuous mercury arc lamp, were used to photorelease InsP_3 from the caged precursor loaded into the oocyte. The relative amount of InsP_3 formed was varied using an electronic shutter to regulate the duration of light flashes and neutral density filters to control intensity. The photolysis light was introduced through the epifluorescence port of the microscope and was focused uniformly as a 200-μm diameter spot on the cell, centered around and parafocal with the laser scan line. For experiments with Ca^{2+} injections, oocytes were not loaded with caged InsP_3 or indicator, and injections were made by applying pneumatic pressure pulses to a third micropipette filled with 0.5 M $CaCl_2$ while the cell was voltage clamped (13, 18). All experiments were done in the animal hemisphere of oocytes, because this contains a higher density of Ca^{2+}-activated channels (10, 13) and InsP_3-sensitive Ca^{2+} release sites (6).

Fluorescence signals and currents were quantified by measuring the amplitudes of their respective peaks. We did not attempt to correlate simultaneous measurements of current and fluorescence, because of uncertainties in their kinetic relationship (e.g., delays due to Ca^{2+} diffusion and Cl^- channel activation). However, the peak current was usually attained shortly (ca 100 ms) following the peak fluorescence, at a time when the fluorescence had declined only slightly (<10%). To compare responses at different membrane potentials, measurements of currents were expressed as membrane conductance, so as to normalize for ohmic changes in current and better reveal the voltage dependence of activation of $I_{Cl,Ca}$. Conductances were calculated using measured values of reversal potentials for $I_{Cl,Ca}$, which were close to −20 mV, corresponding to the Cl^- equilibrium potential in the oocyte (13). Outward currents at voltages positive to this potential are thus carried by an influx of Cl^-, and inward currents at more negative potentials by efflux of Cl^-.

In addition to Cl^- currents evoked by InsP_3-mediated Ca^{2+} liberation, oocytes from some donor frogs display Cl^- currents on depolarization due to activation of voltage-dependent membrane Ca^{2+} channels (13). These latter currents were absent or negligible (<50 nA) in the oocytes used for the present experiments. Furthermore, they would not have complicated measurements of InsP_3-evoked signals, because the Cl^- currents generated by Ca^{2+} entry through endogenous voltage-gated channels decay within about 2 s (13), whereas photolysis flashes were delivered after the oocytes had been held at a given potential for >10 s.

OG-5N and caged InsP_3 were obtained from Molecular Probes (Eugene, OR). All other reagents were from Sigma Chemical (St. Louis, MO).

RESULTS

Cl^- currents and Ca^{2+} fluorescence signals evoked by photoreleased InsP_3. Figure 1 shows records of Ca^{2+} fluorescence signals (*top traces*) and currents (*bottom traces*) obtained at clamp potentials of −160 mV (Fig. 1*A*) and +40 mV (Fig. 1*B*) in a single oocyte in response to photorelease of InsP_3 by UV light flashes of increasing durations. These data are representative of results obtained in a total of 12 oocytes. As we have described previously, Ca^{2+} waves (22) and Ca^{2+}-activated currents (18) were evoked only by flashes exceeding a certain threshold duration. With suprathreshold stimuli, the amplitudes of the Ca^{2+} signals then increased progressively with increasing flash duration and were similar at the two holding potentials. In contrast, the corresponding membrane currents differed markedly at negative and positive potentials. Inward currents at −160 mV were brief and varied progressively in magnitude with the size of the underlying Ca^{2+} transient. Outward currents at +40 mV, on the other hand, were slower and exhibited a steeper dose-response characteristic.

Measurements of peak fluorescence signals and currents are shown plotted as a function of flash duration in Figs. 1, *C* and *D*. At −160 mV the current amplitude increased in a graded manner as the flashes were progressively lengthened. In contrast, currents at +40 mV exhibited a lower threshold and increased more steeply with increasing flash duration so as to more rapidly attain a maximum, saturating value. Maximal currents at +40 mV were smaller than at −160 mV, but this could be attributed simply to the different electrical driving force for Cl^- flux through the channels. To eliminate the ohmic voltage dependence of the current,

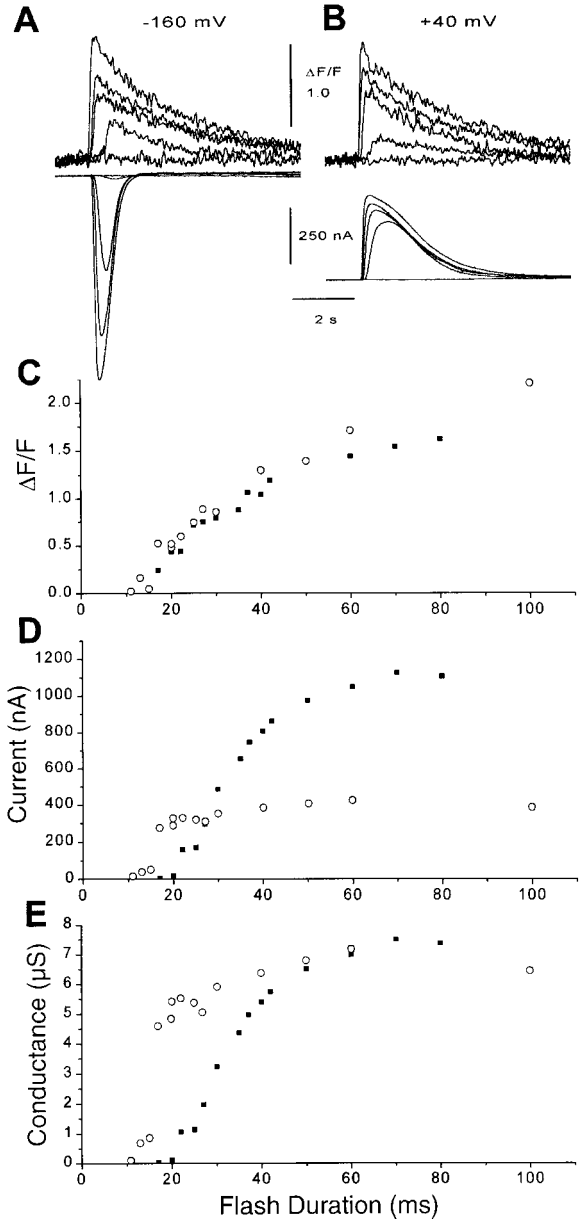

Fig. 1. Membrane currents and Ca^{2+} signals evoked at holding potentials of −160 mV and +40 mV by photorelease of increasing amounts of inositol 1,4,5-trisphosphate (InsP_3). Data are shown from single oocyte, and are representative of results in 12 cells. *A* and *B*: responses evoked by photolysis flashes with durations of 13, 20, 30, 40, and 60 ms. *Top traces*: Ca^{2+}-dependent fluorescence signals, averaged over 50-μm-long confocal scan line centered within photolysis spot. *Bottom traces*: corresponding membrane currents. Oocyte was clamped at potentials of −160 mV (*A*) and +40 mV (*B*), and records were obtained only after holding at given potential for >10 s. Oocyte was bathed in normal Ringer solution (1.8 mM $CaCl_2$). *C*: measurements of peak Oregon Green 488 1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-5N (OG-5N) fluorescence signals at holding potentials of −160 mV (■) and +40 mV (○). *D*: corresponding measurements of absolute peak Ca^{+}-activated Cl^- currents ($I_{Cl,Ca}$). *E*: conductance changes evoked by photolysis flashes, calculated from data in *D*, using value of −16 mV for the reversal potential of $I_{Cl,Ca}$ measured in this cell. ΔF/F, ratio relative to resting fluorescence before stimulation.

the data are expressed as membrane conductance in Fig. 1*E*. This displays more clearly the differing dose dependence of activation of $I_{Cl,Ca}$ at positive and negative potentials and shows that the maximal conductances at high [InsP_3] were similar at positive and negative potentials. The different dose dependence was not due to differences in [Ca^{2+}] transients, because the corresponding fluorescence signals showed closely similar peak amplitudes at positive and negative holding potentials (Fig. 1*C*). Instead, the differences in dose dependence likely arise through voltage-dependent changes in Ca^{2+} activation of the Cl^- channels.

A possible concern, however, was whether Ca^{2+} influx across the plasma membrane may have locally activated Cl^- channels without producing appreciable fluorescence signals. This is unlikely because similar data (not shown) were obtained in four oocytes bathed in Ca^{2+}-free Ringer solution. Furthermore, InsP_3-mediated activation of store-operated Ca^{2+} influx develops much more slowly than the transient responses evoked here (28) and, in any case, is not expected to contribute to the increased sensitivity of $I_{Cl,Ca}$ to Ca^{2+} at positive potentials because the electrochemical gradient for Ca^{2+} entry is reduced at these voltages.

Density of Ca^{2+}-activated Cl^- channels. Figure 2, inset, shows the mean values of currents evoked by supramaximal photolysis flashes (intensities >50 times threshold) in oocytes from five different donor frogs. Measurements were made at a holding potential of +40 mV, using a 200-μm-diameter photolysis spot positioned near the animal pole. The maximal currents were relatively consistent within oocytes obtained from a given donor frog but varied more widely between oocytes from different frogs. Among the donors examined, mean currents ranged from ~300–1,200 nA. The overall current was 843 ± 66 (SE) nA (35 oocytes), which corresponds to a conductance of 14.05 μS (assuming a Cl^- equilibrium potential of −20 mV). Given the restricted spatial photorelease of InsP_3 (200-μm-diam spot), the maximal conductance was ~600 pS per square micrometer of cell surface (neglecting membrane infoldings) and the maximal whole cell conductance from the entire oocyte is predicted to be about 1,200 μS, assuming a diameter of 1.2 mm and a twofold lower density of Cl^- channels in the vegetal hemisphere (6). The single channel conductance of Ca^{2+}-activated Cl^- channels in the oocyte membrane measured by patch-clamp studies is about 3 pS (27), indicating that the channel density in the animal hemisphere is roughly 200/μm^2 considering the oocyte as a smooth sphere, a value that may be reduced as much as 10-fold in terms of density per square micrometer of actual membrane area because of the numerous microvilli (8).

Relationship between $I_{Cl,Ca}$ and cytosolic [Ca^{2+}]. The relationship between the cytosolic Ca^{2+} signal and activation of $I_{Cl,Ca}$ was determined from experiments such as that in Fig. 1, using a range of flash strengths to evoke responses of varying magnitudes while fluorescence transients were imaged with the confocal scan line focused within 2 μm of the cell surface. Figure 2*A* shows pooled measurements from 12 oocytes, plotting

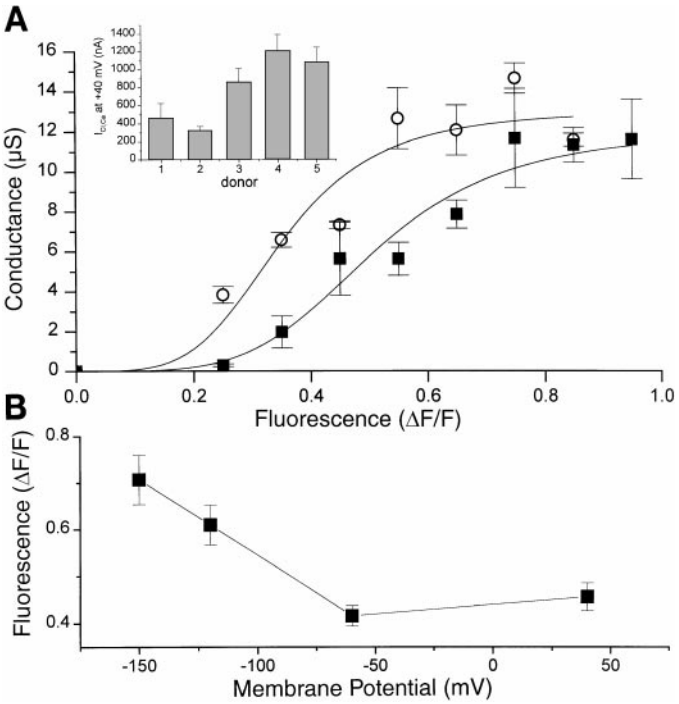

Fig. 2. Activation of $I_{Cl,Ca}$ as function of cytosolic Ca^{2+} concentration ($[Ca^{2+}]$). *Inset*: histogram shows mean currents evoked at holding potential of +50 mV in oocytes from 5 different donor frogs in response to supramaximal photolysis flashes. Error bars, SD. *A*: relationship between peak OG-5N fluorescence signal and corresponding mean peak Cl^- conductance. Measurements were made at holding potentials of +40 mV (○) and −150 mV (■) in response to photolysis flashes of varying durations and intensities. Data are from 12 oocytes and show mean conductances ± SE, corresponding to fluorescence signals binned at increments of 0.1 ΔF/F. Curves are Hill relationships with respective Hill coefficients of 4.2 and 4.39 and half-maximal activation of 0.35 ΔF/F and 0.51 ΔF/F, respectively, for the +40 mV and −150 mV data. *B*: magnitudes of fluorescence signals evoked by photolysis flashes that produced half-maximal Cl^- currents at various holding potentials. Points show means ± SE of peak OG-5N signals measured in 5 oocytes. Data were obtained by first determining peak $I_{Cl,Ca}$ evoked at each potential by a supramaximal photolysis flash and then adjusting flash strength to produce current of one-half that amplitude.

the peak conductance change at holding potentials of +40 and −150 mV as a function of OG-5N fluorescence ratio. The maximal conductance at high $[Ca^{2+}]$ was similar at both voltages, but the relationship at −150 mV was shifted to the right compared with that at +40 mV so that intermediate Ca^{2+} levels resulted in smaller conductance changes at the hyperpolarized potential. The fluorescence signal associated with half-maximal activation of $I_{Cl,Ca}$ increased by about 46% on polarization from +40 to −150 mV, but a much more pronounced effect was evident at low Ca^{2+} levels. For example, the conductance change associated with a fluorescence signal of 0.25 ΔF/F was nearly 20-fold greater at +40 mV than at −150 mV.

Measurements of fluorescence signals corresponding to half-maximal Cl^- currents at various voltages are shown in Fig. 2*B* and provide an estimate of how the apparent affinity for Ca^{2+} activation of the Cl^- conductance varies with voltage. Little difference was apparent between +40 mV and −60 mV, but the Ca^{2+} levels associated with half-maximal activation then increased progressively at more negative potentials and were about 57% greater at −150 mV compared with +40 mV. From the calibration factor given in METHODS, the fluorescence ratio values in Fig. 2*B* correspond to free Ca^{2+} concentrations of about 2.1 μM at +40 and −60 mV, 3 μM at −120 mV, and 3.5 μM at −150 mV.

Voltage dependence of InsP_3-evoked currents and Ca^{2+} signals. To explore further the interaction between voltage and $[Ca^{2+}]$ in the activation of $I_{Cl,Ca}$, we recorded fluorescence signals and membrane currents evoked by photorelease of InsP_3 while the membrane potential was clamped over a range of holding voltages. Figure 3 shows representative records from an oocyte stimulated by photolysis flashes that were relatively strong (135 ms; Fig. 3*A*) or weak (35 ms; Fig. 3*B*) in relation to the threshold flash duration required to evoke $I_{Cl,Ca}$ at +40 mV (about 25 ms). The InsP_3-evoked Ca^{2+} signals changed relatively little with voltage, other than a slight acceleration of their decay at more positive potentials. In contrast, the amplitudes and kinetics of $I_{Cl,Ca}$ changed markedly with voltage. Currents evoked by both strong and weak flashes reversed direction at about −18 mV in this oocyte, and at increasingly positive potentials, the currents became slower and exhibited a prolonged plateau. The peak amplitudes of outward currents evoked by both weak and strong flashes increased progressively as the membrane potential was clamped at voltages increasingly positive to the reversal potential. At more negative potentials, however, responses to strong flashes differed markedly from those evoked by weak flashes. Inward currents evoked by the 35-ms flashes increased only slightly or even declined with hyperpolarization beyond about −60 mV, despite the increased electrical driving force for Cl^- efflux (Fig. 3*B*). In contrast, the peak amplitudes of currents evoked by 135-ms flashes continued to increase progressively with hyperpolarization to at least −140 mV (Fig. 3*A*).

I-V relationships (Fig. 3*C*) derived from these data showed a nearly linear voltage dependence for $I_{Cl,Ca}$ evoked by the 135-ms flash, whereas currents evoked by the 35-ms flash showed a marked outward rectification. The ratio of the current amplitudes evoked by the strong and weak flashes varied from about 1.5 at a potential of +50 mV to about 4 at −180 mV. This shift from an outwardly rectifying to a linear *I-V* relationship with increasing photorelease of InsP_3 was consistently observed in all oocytes examined (12 cells; 5 donor frogs).

The outward rectification of currents evoked by weak flashes was not due to decreased Ca^{2+} liberation at more negative potentials, because the corresponding fluorescence signals changed little or not at all at voltages between −180 and +60 mV (Fig. 3*D*). Instead, we interpret the differing shapes of the *I-V* relationships with weak and strong stimuli to arise through the voltage-dependent decrease in sensitivity of the Cl^- conductance to Ca^{2+} at more negative potentials. Thus intracellular free $[Ca^{2+}]$ levels resulting from the strong flash were sufficient to maximally activate the conduc-

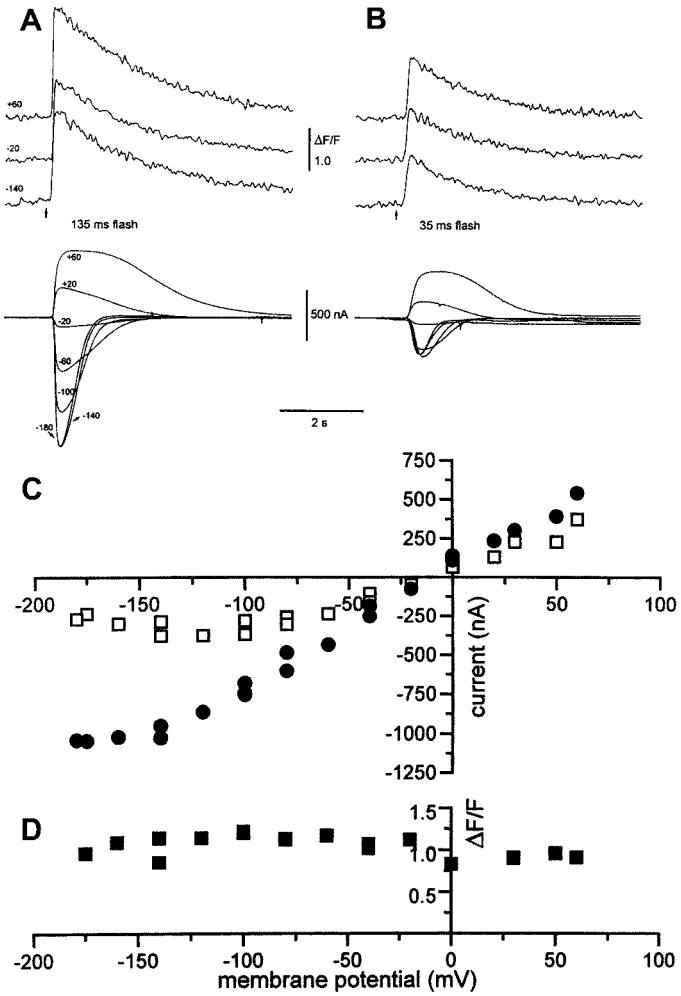

Fig. 3. Fluorescence Ca^{2+} signals and $I_{Cl,Ca}$ evoked by strong and weak photorelease of InsP_3 while clamping at various membrane potentials. *A* and *B*: traces show OG-5N fluorescence (*top, noisy traces*) and membrane current (*bottom traces*) in response to photolysis flashes. Flash duration in *A* (135 ms) was >5 times the threshold required to evoke a Ca^{2+} wave (about 25 ms), whereas the flash duration in *B* (35 ms) was only slightly above threshold. Superimposed current traces in both frames show $I_{Cl,Ca}$ evoked by flashes at potentials (mV) indicated in *A*. Records were obtained after oocyte had been clamped at given potential for >10 s, and are shown after subtracting holding current. Fluorescence signals exhibited relatively little variation at different holding potentials, and examples are illustrated at only 3 representative potentials for clarity (indicated in mV). *C*: peak amplitudes of membrane currents evoked by photolysis flashes with durations of 35 ms (*open symbols*) and 135 ms (*solid symbols*) as function of clamp potential. Data are from same oocyte in *A* and are representative of experiments in a total of 12 cells. *D*: peak amplitudes of Ca^{2+} fluorescence signals evoked by the 35-ms flashes show little or no voltage dependence.

tance at all voltages between approximately +60 and −140 mV, whereas currents evoked by the weak flashes diminished at increasingly hyperpolarized potentials as the sensitivity of the Cl^- conductance became progressively lower in relation to the smaller cytosolic [Ca^{2+}] transient.

In accordance with this interpretation, the shape of the *I-V* relationship depended on the current density (current per unit area of membrane) rather than on the absolute whole cell current. For example, when the photolysis light was focused sharply as a 200-μm diameter spot, a flash of 60-ms duration evoked currents with a linear voltage dependence. After deliberately defocusing the light to illuminate a broader area of the cell, currents evoked by flashes of the same duration then exhibited a pronounced outward rectification, so that the current at −130 mV was only 55% of that with the sharply focused spot, even though the currents at +30 mV were similar (2 oocytes examined).

Voltage-dependent kinetics of Ca^{2+} transients and $I_{Cl,Ca}$. As illustrated in Fig. 3, *A* and *B*, the kinetics of $I_{Cl,Ca}$ varied strongly with holding potential, although the time course of the corresponding Ca^{2+} signals exhibited only slight voltage dependence. To quantify these effects, we measured the time to fall to one-half the peak value ($t_{1/2}$) of currents and fluorescence signals evoked by supramaximal photolysis flashes over a range of holding potentials (Fig. 4*A*). The decay of the

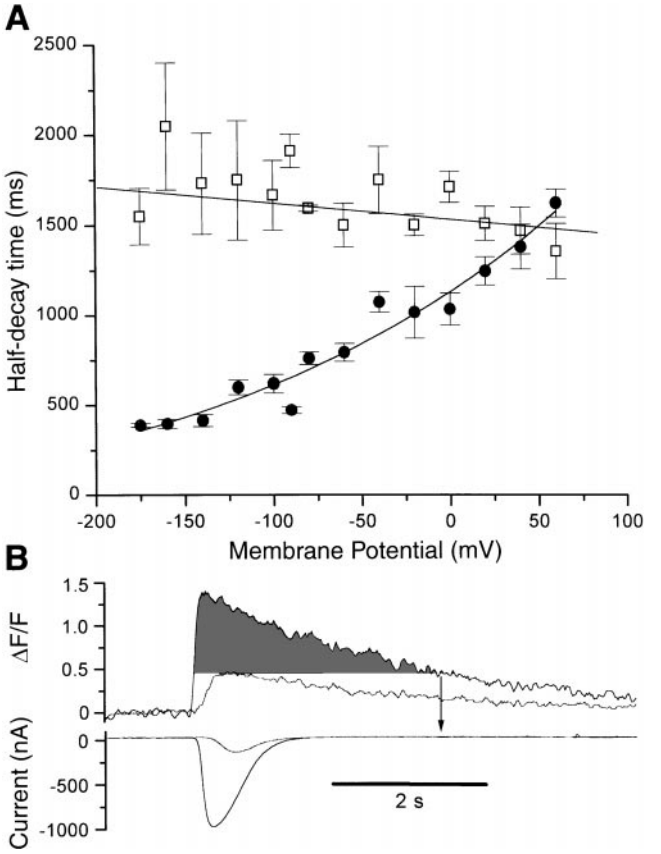

Fig. 4. Differing voltage dependence of Ca^{2+} signals and $I_{Cl,Ca}$. *A*: data show decay times of fluorescence Ca^{2+} signals (□) and Cl^- currents (●) evoked by supramaximal photorelease of InsP_3 as function of holding potential. Measurements were made of times to decay to one-half peak value, and points show means ± SE from >3 oocytes (2 donors). Regression line is fitted to fluorescence data. Curve fitted to data for half-decay times of $I_{Cl,Ca}$ is exponential with slope of 211 mV per *e*-fold change in $t_{1/2}$. *B*: rapid decay of $I_{Cl,Ca}$ at negative potentials is not explained simply by reduced sensitivity to Ca^{2+}. Superimposed traces show fluorescence signals (*top*) and currents (*bottom*) evoked in same oocyte by photolysis flashes with durations of 22 and 50 ms. Fluorescence signal evoked by strong flash remained above peak level evoked by weak flash for about 3 s (shaded area), but corresponding current declined to baseline well before end of this time (arrow).

currents shortened markedly at increasingly negative potentials, with $t_{1/2}$ decreasing as an exponential function of voltage from about 1.6 s at +60 mV to 350 ms at −175 mV. In contrast, the voltage dependence of decay of the fluorescence signals was more shallow and in the opposite direction; the $t_{1/2}$ lengthened from about 1.5 s at +60 mV to about 1.75 s at strongly negative potentials.

The increasing discrepancy in time course between the Cl^- currents and Ca^{2+} signals at more negative potentials was not due simply to a reduction in Ca^{2+} sensitivity, such that the current terminated more rapidly because the Ca^{2+} level, as indicated by the fluorescence signal, declined below the higher threshold level needed for activation of $I_{Cl,Ca}$. This is illustrated in Fig. 4*B*, which compares fluorescence and current signals evoked at −160 mV by photolysis flashes with durations of 22 and 50 ms. The current evoked by the stronger flash terminated rapidly, and no current was evident during an appreciable time for which the fluorescence signal remained higher than the peak level evoked by the weaker flash, to which there was a clear current response.

Graded rectification of $I_{Cl,Ca}$ as a function of stimulus strength. Figure 5*A* plots pooled data from five oocytes (3 donor frogs), showing that the form of the *I-V* relationship of $I_{Cl,Ca}$ varied progressively from almost complete outward rectification with just suprathreshold photolysis flashes to near perfect linearity with very strong flashes. Normalized Cl^- conductance changes derived from these data are shown in Fig. 5*B*, to remove the ohmic voltage dependence of the current and display more clearly the effect of voltage on activation of $I_{Cl,Ca}$. The strongest flash was >100 times stronger than the threshold required to evoke a current at +40 mV and produced a conductance that remained constant between +40 and −160 mV, suggesting that cytosolic $[Ca^{2+}]$ was sufficiently high to maximally activate the channels even at strongly hyperpolarized potentials. In contrast, the conductance change evoked by the weakest flash (~1.2 times threshold) declined rapidly at potentials negative to +40 mV and decreased to ~5% at −120 mV. Flashes of intermediate strengths gave intermediate conductance-voltage relationships.

Instantaneous and steady-state I-V relationships. The results presented above are consistent with $I_{Cl,Ca}$ arising through channels, the gating of which is determined by $[Ca^{2+}]$ and modulated by voltage and which exhibit an ohmic open channel *I-V* relationship (as demonstrated by the linear voltage dependence with strong stimuli). To confirm this latter point, we measured instantaneous *I-V* relationships (reflecting current flow through open channels) as well as steady-state relationships (reflecting both probability of channel opening and open channel current). Figure 6 shows data from an oocyte stimulated by relatively weak (1.5 times threshold at +20 mV) and stronger (7 times threshold) photolysis flashes. Steady-state current measurements were obtained, as in Fig. 2, by delivering photolysis flashes after clamping at a given potential for several seconds. To determine instantaneous currents, flashes were delivered with the oocyte clamped at +20 mV and the potential was stepped to more negative levels at about the time of the peak current (800 ms following the flash). Measurements of instantaneous currents were made about 8 ms after the voltage step, to allow time for capacitative currents to settle, and are presented after subtraction of passive currents evoked by equivalent voltage steps in the absence of photolysis flashes. The steady-state *I-V* relationship for the weak flash exhibited characteristic outward rectification, whereas the corresponding instantaneous *I-V* relationship was close to linear. With the strong flash, on the other hand, both the instantaneous and steady-state relationships were linear and closely similar.

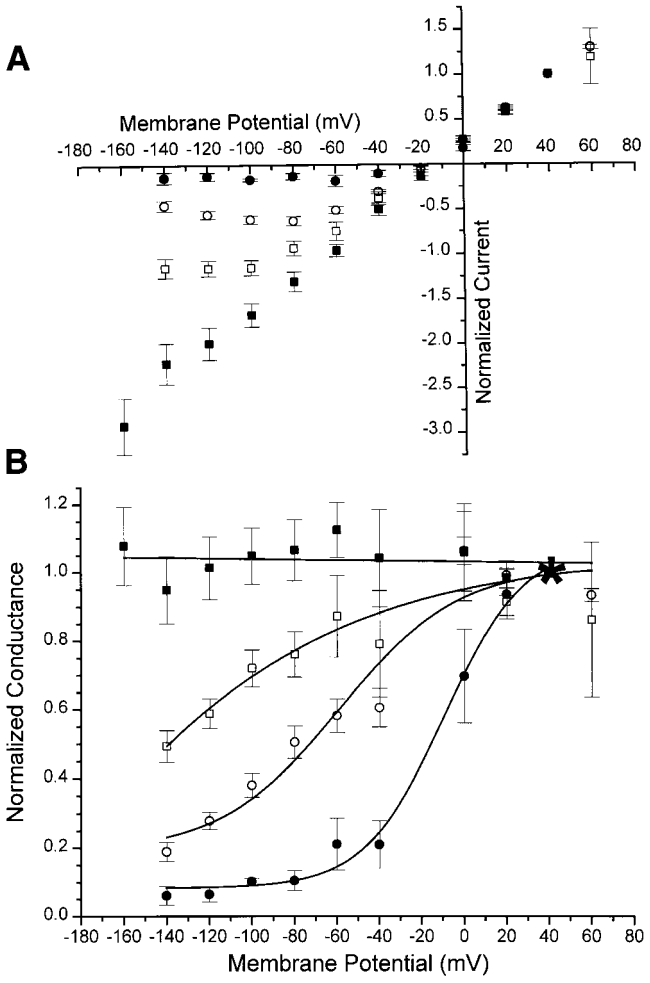

Fig. 5. $I_{Cl,Ca}$ shows graded transition from strong outward rectification to linear relationship with increasing photorelease of $InsP_3$. *A*: current-voltage (*I-V*) relationships obtained for responses evoked by photolysis flashes with durations of ~140% (●), 180% (○), 260% (□), and >1,000% (■) relative to threshold required to evoke $I_{Cl,Ca}$ at +40 mV. Currents are shown normalized relative to mean current evoked by each flash duration at +40 mV. Data points represent means ± SE from 5 oocytes. *B*: plots of $InsP_3$-evoked conductance as function of membrane potential, derived from data in *A*.

Kinetics of Ca^{2+}-sensitivity changes. To examine the rapidity with which the Ca^{2+} sensitivity of the Cl^- conductance changed with potential, we measured the relaxation times of tail currents following voltage steps.

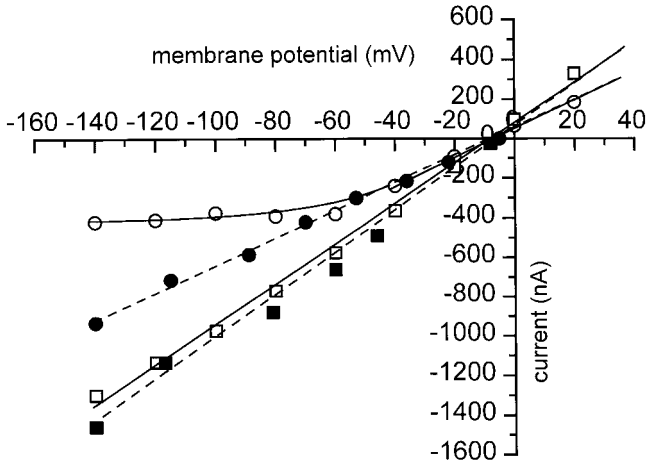

Fig. 6. Steady-state and instantaneous *I-V* relationships for $I_{Cl,Ca}$ evoked by weak and strong photolysis flashes. Open symbols show steady-state currents evoked by photolysis flashes with durations of 150% (○) and 700% (□) relative to threshold measured as in Fig. 2. Solid symbols indicate corresponding instantaneous currents. Oocyte was held at potential of +20 mV while photolysis flash was delivered, and voltage was then stepped to various levels just after peak $I_{Cl,Ca}$ was attained. Measurements of instantaneous current were made ~8 ms after voltage step (to allow for decay of capacitative transient) and were plotted after subtraction of passive currents.

These measurements were not readily obtained from responses evoked by photoreleased InsP_3, due to their rapid decay (e.g., Fig. 1). Instead, we applied voltage pulses during submaximal currents evoked by microinjecting Ca^{2+} into the oocyte through a pipette filled with 0.5 M $CaCl_2$, which persist for a few seconds (13). Hyperpolarizing steps applied from a holding potential of −50 mV evoked large instantaneous inward currents that decayed rapidly, whereas positive-going steps evoked smaller instantaneous currents followed by a more slowly increasing outward current (Fig. 7*A*). Similar to the *I-V* relationships obtained with InsP_3-evoked responses, the steady-state currents measured following Ca^{2+} injections exhibited outward rectification (Fig. 7*B*), whereas instantaneous currents were more closely linear. The half-times of the current relaxations lengthened from ~20 ms at −160 mV to nearly 100 ms at +10 mV (Fig. 7*C*).

DISCUSSION

We found that Cl^- currents in *Xenopus* oocytes activated by InsP_3-mediated liberation of intracellular Ca^{2+} exhibited a marked increase in sensitivity to $[Ca^{2+}]$ at increasingly positive membrane potentials. Consequently, the shape of the *I-V* relationship varied with $[Ca^{2+}]$. Currents evoked by small Ca^{2+} elevations exhibited outward rectification, with little or no response at strongly negative potentials, whereas a linear *I-V* relationship was obtained with Ca^{2+} elevations sufficiently large to cause maximal activation over a wide voltage range. These findings are most easily explained by the presence of a single class of Ca^{2+}-activated Cl^- channels that exhibit a voltage-dependent activation by cytosolic Ca^{2+} and a voltage-independent open-channel conductance. The idea that there are two or more classes of Cl^- channels with differing Ca^{2+} affinities (11) appears less plausible, because our data indicate that this would require both outwardly rectifying channels with high Ca^{2+} affinity and inwardly rectifying channels with low affinity, present at an appropriate relative density in the oocyte membrane so as to result in an overall linear *I-V* relationship with strong Ca^{2+} activation. Our results

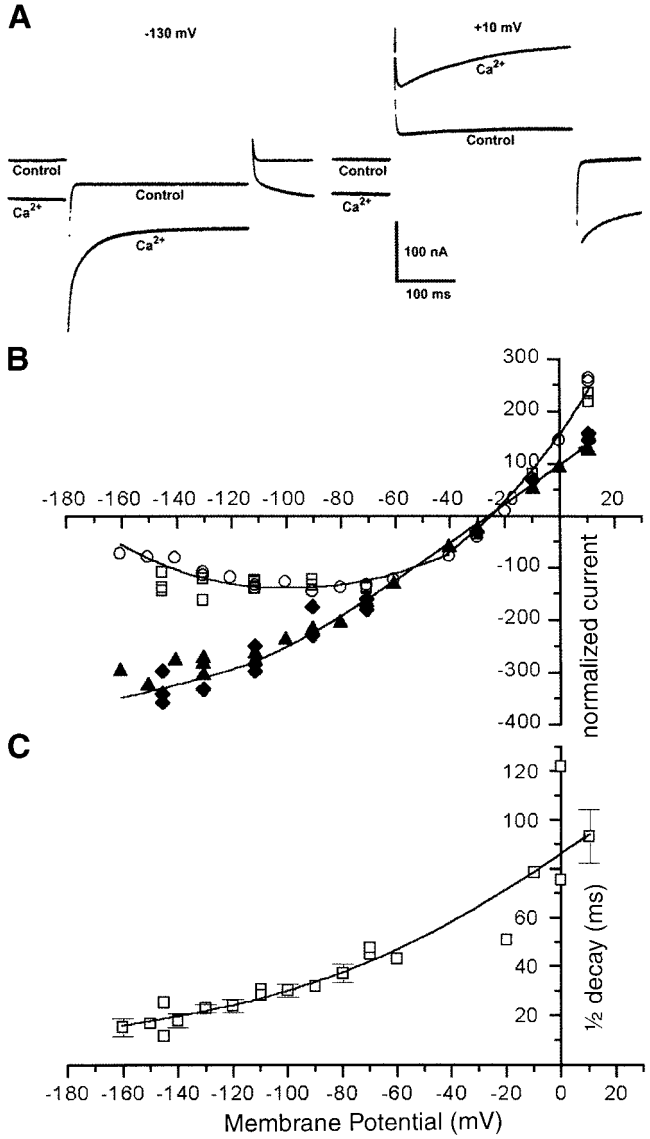

Fig. 7. Voltage-dependent relaxations of $I_{Cl,Ca}$, measured by applying voltage steps during currents evoked by intracellular Ca^{2+} injections. *A*: relaxations of $I_{Cl,Ca}$ following voltage steps. Injections of Ca^{2+} were made with membrane potential clamped at −50 mV, and voltage was then stepped to indicated values at about the time of maximal current activation. Each frame contains 2 superimposed records, showing currents evoked by identical voltage steps with and without prior injection of Ca^{2+}. *B*: *I-V* relationships derived from records like those in *A*. Measurements are expressed as percentage of peak $I_{Cl,Ca}$ at −50 mV, just before delivery of voltage step, and are plotted after subtraction of passive currents. Solid symbols indicate instantaneous currents measured ~8 ms after voltage step, and open symbols indicate steady-state currents at end of 250-ms-duration voltage steps. Data are from 2 oocytes (indicated by squares and circles). *C*: voltage-dependence of half-relaxation time of $I_{Cl,Ca}$, derived from records like those in *A*. Data are from 2 oocytes.

and conclusion are similar, however, to a recent study by Kuruma and Hartzell (12), who employed Ca^{2+} injections and Ca^{2+} influx through heterologously expressed Ca^{2+} channels to show that outward Cl^- currents in the oocyte are more sensitive to Ca^{2+} than inward currents. In that study they also described apparent differences in anion selectivity and instantaneous *I-V* relationships between Cl^- currents activated by different voltage-clamp protocols but concluded that these may arise through experimental limitations and that the hypothesis that a single type of Cl^- channel remained valid (12).

In agreement with Kuruma and Hartzell (12), we believe a likely explanation for the voltage-dependent sensitivity of the Cl^- current to cytosolic Ca^{2+} is that the apparent affinity of the channel for Ca^{2+} is voltage dependent, a property that is common among Ca^{2+}-activated membrane channels. For example, Ca^{2+}-activated Cl^- channels in rat parotid acinar cells (1) and in pulmonary artery endothelial cells (15) exhibit increased activation at more positive potentials, as do large-conductance Ca^{2+}-activated K^+ channels (9). The increasing sensitivity of the Cl^- current at more positive potentials may be due to Ca^{2+} remaining bound to the channel for a longer time, and in agreement relaxations of $I_{Cl,Ca}$ following voltage steps occur with a half-time that lengthens from about 20 ms at −160 mV to about 100 ms at +10 mV. These values are closely similar to those obtained for half-times of deactivation of $I_{Cl,Ca}$ in excised patches of oocyte membrane following removal of Ca^{2+} (10), suggesting that the voltage-dependent changes in affinity of the Cl^- channel arise largely through changes in off-rate for Ca^{2+} dissociation rather than in the rate of Ca^{2+} binding. Our measurements, however, represent population behavior of the underlying channels and do not allow a precise molecular interpretation. It therefore remains possible that processes other than changes in affinity may account for the voltage-dependent sensitivity of the Cl^- current (e.g., changes in channel gating subsequent to Ca^{2+} binding; modulation of local cytosolic [Ca^{2+}] gradients near the plasma membrane). Detailed kinetic studies employing patch-clamp recording are required to unambiguously resolve these issues.

Fluorescence measurements of the intracellular free Ca^{2+} transients underlying the Cl^- currents indicated that half-maximal activation of the Cl^- conductance required ~3.5 μM Ca^{2+} at −150 mV and about 2 μM Ca^{2+} at +40 mV. These values are within the range of cytosolic Ca^{2+} concentrations attained during InsP_3-mediated signaling (22), and even at strongly negative potentials sufficient Ca^{2+} could be liberated in most oocytes by strong photorelease of InsP_3 to maximally activate the conductance. However, the voltage-dependent shift in the Ca^{2+} activation of $I_{Cl,Ca}$ (Fig. 2*B*) results in much more prominent effects at low [Ca^{2+}] than suggested by the relatively modest change in concentrations required for half-maximal activation. For example, conductances evoked by just suprathreshold photorelease of InsP_3 decreased by almost 95% with polarization from +40 to −140 mV. This large change may account for observations that intracellular Ca^{2+} elevations preferentially activate outward Cl^- currents (12) and that Ca^{2+} influx (which is expected to produce a high, local [Ca^{2+}] near the plasma membrane) readily activates inward currents at hyperpolarized potentials, whereas Ca^{2+} liberation from more distant intracellular stores is relatively ineffective (11, 12).

Gomez-Hernandez et al. (10) reported that a concentration of ~27 μM Ca^{2+} was required for 40% activation of Cl^- current in excised patches of *Xenopus* oocyte membrane. One explanation for the apparent discrepancy between this value and our results may be that the sensitivity of the channels is higher in the intact oocyte compared with an excised patch. Alternatively, high Ca^{2+} levels may be sensed by the Cl^- channels if they are located close to InsP_3-sensitive Ca^{2+} release sites but would not be accurately reflected in the fluorescence measurements. If this is the case, such local Ca^{2+} gradients must be very narrowly delineated, as our fluorescence measurements were obtained from a confocal section <1 μm thick (17) focused within about 2 μm of the plasma membrane, and we did not observe larger signals closer to the plasma membrane (5).

A further unresolved question concerns the kinetic relationship between Ca^{2+} signals and $I_{Cl,Ca}$. The decay of InsP_3-evoked fluorescence signals exhibited a slight slowing with polarization to more positive potentials, possibly because the mechanisms extruding Ca^{2+} across the plasma membrane functioned more effectively because of the reduced electrochemical gradient for Ca^{2+} across the membrane. In contrast, the decay of Cl^- currents lengthened considerably at increasingly positive potentials, following an exponential relationship with an *e*-fold change in $t_{1/2}$ per 210 mV. The increasing discrepancy between time courses of the fluorescence signal and $I_{Cl,Ca}$ with hyperpolarization is not readily explained as a simple consequence of the reduced sensitivity to Ca^{2+} but suggests that the kinetics of termination of the Cl^- conductance are voltage sensitive. The reason why $I_{Cl,Ca}$ declines more rapidly than the Ca^{2+} signal at negative voltages, however, remains unclear. We had previously proposed that the Cl^- channels exhibit an adaptive behavior to Ca^{2+} (21), but subsequent findings of sustained Cl^- currents evoked by Ca^{2+} application to excised membrane patches (10) and by photolysis of caged Ca^{2+} in intact oocytes (unpublished data) make this idea less tenable. Instead, a close coupling between Ca^{2+} release sites and Cl^- channels may help explain the dissociation in time courses of the confocal fluorescence signal and $I_{Cl,Ca}$.

The high sensitivity of the Cl^- conductance to [Ca^{2+}] at positive membrane potentials may assist in experiments where voltage-clamp recording of Cl^- current is used as a convenient, endogenous monitor of intracellular Ca^{2+}. Furthermore, it is likely to be of physiological importance in the generation of the fertilization potential that results from an InsP_3-dependent wave of intracellular Ca^{2+} spreading from the sperm entry site (16). The Cl^- equilibrium potential for eggs in pond water is positive, so that opening of Cl^- channels

results in a depolarization. This is likely to be reinforced robustly by the regenerative characteristic imparted by the increasing sensitivity to intracellular Ca^{2+} at increasingly positive potentials.

We thank Dr. Jennifer Kahle for editorial assistance. This work was supported by the National Institute of General Medical Sciences Grant GM-48071.

Address for reprint requests and other correspondence: I. Parker, Laboratory of Cellular and Molecular Neurobiology, Dept. of Neurobiology and Behavior, Univ. of California, Irvine, CA 92697 (E-mail: iparker@uci.edu).

Received 16 August 1999; accepted in final form 26 October 1999.

REFERENCES

1. **Arreola J, Melvin JE, and Begenisich T.** Activation of calcium-dependent chloride channels in rat parotid acinar cells. *J Gen Physiol* 108: 35–47, 1996.
2. **Berridge MJ.** Inositol trisphosphate and calcium signaling. *Nature* 361: 315–325, 1993.
3. **Boton R, Dascal N, Gillo B, and Lass Y.** Two calcium-activated chloride conductances in Xenopus laevis oocytes permeabilized with the ionophore A23187. *J Physiol (Lond)* 408: 511–534, 1987.
4. **Callamaras N and Parker I.** Caged inositol 1,4,5-trisphosphate for studying release of Ca^{2+} from intracellular stores. *Methods Enzymol* 291: 497–499, 1998.
5. **Callamaras N and Parker I.** Radial localization of InsP_3-sensitive Ca^{2+} release sites in *Xenopus* oocytes resolved by axial confocal linescan imaging. *J Gen Physiol* 113: 199–213, 1999.
6. **Callamaras N, Sun X-P, Ivorra I, and Parker I.** Hemispheric asymmetry of macroscopic and elementary calcium signals mediated by InsP_3 in *Xenopus* oocytes. *J Physiol (Lond)* 511: 395–405, 1998.
7. **Cross NL and Elinson RP.** A fast block to polyspermy in frogs mediated by changes in the membrane potential. *Dev Biol* 75: 187–198, 1980.
8. **Dascal N.** The use of *Xenopus* oocytes for the study of ion channels. *Crit Rev Biochem* 22: 317–387, 1987.
9. **DiChiara TJ and Reinhart PH.** Distinct effects of Ca^{2+} and voltage on the activation and deactivation of cloned Ca^{2+}-activated K^+ channels. *J Physiol (Lond)* 489: 403–418, 1995.
10. **Gomez-Hernandez J-M, Stuhmer W, and Parekh AB.** Calcium-dependence and distribution of calcium-activated chloride channels in *Xenopus* oocytes. *J Physiol (Lond)* 502: 569–574, 1997.
11. **Hartzell HC.** Activation of different Cl currents in *Xenopus* oocytes by Ca liberated from stores and by capacitative Ca influx. *J Gen Physiol* 108: 157–175, 1996.
12. **Kuruma A and Hartzell HC.** Dynamics of calcium regulation of chloride currents in *Xenopus* oocytes. *Am J Physiol Cell Physiol* 276: C161–C175, 1999.
13. **Miledi R and Parker I.** Chloride current induced by injection of calcium into Xenopus oocytes. *J Physiol (Lond)* 357: 173–183, 1984.
14. **Miledi R, Parker I, and Sumikawa K.** Transplanting receptors from brains into oocytes. In: *Fidia Research Foundation Neuroscience Award Lectures.* New York: Raven, p. 57–90, 1989.
15. **Nilius B, Prenen J, Szucs G, Wei L, Tanzi F, Voets T, and Droogmans G.** Calcium-activated chloride channels in bovine pulmonary artery endothelial cells. *J Physiol (Lond)* 498: 381–396, 1997.
16. **Nuccitelli R, Yim DL, and Smart T.** The sperm-induced Ca^{2+} wave following fertilization of the *Xenopus* egg requires the production of Ins(1,4,5)P_3. *Dev Biol* 158: 200–212, 1993.
17. **Parker I, Callamaras N, and Wier WG.** A high-resolution, confocal laser-scanning microscope and flash photolysis system for physiological studies. *Cell Calcium* 21: 441–452, 1997.
18. **Parker I and Ivorra I.** Characteristics of membrane currents evoked by photoreleased inositol trisphosphate in *Xenopus* oocytes. *Am J Physiol Cell Physiol* 263: C154–C165, 1992.
19. **Parker I and Miledi R.** Inositol trisphosphate activates a voltage-dependent calcium influx in Xenopus oocytes. *Proc R Soc Lond B Biol Sci* 231: 27–36, 1987.
20. **Parker I and Miledi R.** Nonlinearity and facilitation in phosphoinositide signaling studied by the use of caged inositol trisphosphate in *Xenopus* oocytes. *J Neurosci* 9: 4068–4077, 1989.
21. **Parker I and Yao Y.** Relation between intracellular Ca^{2+} signals and Ca^{2+}-activated Cl^- current in *Xenopus* oocytes. *Cell Calcium* 15: 276–288, 1994.
22. **Parker I, Yao Y, and Ilyin V.** Fast kinetics of calcium liberation induced in Xenopus oocytes by photoreleased inositol trisphosphate. *Biophys J* 70: 222–237, 1996.
23. **Putney JW.** Inositol phosphates and calcium entry. *Adv Second Messenger Phosphoprotein Res* 26: 143–160, 1992.
24. **Song LS, Sham JS, Stern MD, Lakatta EG, and Cheng H.** Direct measurement of SR release flux by tracking "Ca^{2+} spikes' in rat cardiac myocytes. *J Physiol (Lond)* 512: 677–691, 1998.
25. **Sumikawa K, Parker I, and Miledi R.** Expression of neurotransmitter receptors and voltage-activated channels from brain mRNA in *Xenopus* oocytes. *Methods Neurosci* 1: 30–45, 1989.
26. **Sun X-P, Callamaras N, and Parker I.** A continuum of InsP_3-mediated elementary Ca^{2+} signaling events in *Xenopus* oocytes. *J Physiol (Lond)* 509: 67–80, 1998.
27. **Takahashi T, Neher E, and Sakmann B.** Rat brain serotonin receptors in *Xenopus* oocytes are coupled by intracellular calcium to endogenous channels. *Proc Natl Acad Sci USA* 84: 5063–5067, 1986.
28. **Yao Y and Parker I.** Inositol trisphosphate-mediated Ca^{2+} influx into *Xenopus* oocytes triggers Ca^{2+} liberation from intracellular stores. *J Physiol (Lond)* 468: 275–295, 1993.