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Properties of the Ca^{2+}-activated Cl^- current of *Xenopus* oocytes

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Abstract. The properties of the Ca^{2+}-activated Cl^- current of *Xenopus* oocytes have been investigated by voltage-clamp and injections of D-3-deoxy-3-fluoro-myo-inositol 1,4,5-trisphosphate (3-F-$InsP_3$). Following 3-F-$InsP_3$ injection, a transient phase of Ca^{2+}-activated Cl^- current occurred, caused by Ca^{2+} release from internal stores; subsequently, a secondary, long-lasting, current was recorded, signaling Ca^{2+} influx from the exterior (I_{CRAC}). Changes in external Cl^- during the sustained phase produced the expected shifts in reversal potential (E_{rev}), while the conductance varied opposite to the predictions of simple electrodiffusional theory. Application of depolarizing pulses soon (10 s) after 3-F-$InsP_3$ injection elicited membrane currents exhibiting a single exponential rise. During the sustained subsequent phase, the current elicited by depolarizations showed an early peak followed by a prominent decline. During the sustained phase, removal of calcium from the external solution, or its substitution with Ba^{2+}, abolished voltage- and time-dependent components of the depolarization-induced current. Slope conductance analysis of the inactivating records revealed, in addition to the decline of the Ca^{2+}-activated Cl^- current, the presence of a second, inwardly directed current. This could be identified as a slowly inducible Na^+ current already described in *Xenopus* oocytes.
Key words. Calcium-dependent chloride current; *Xenopus* oocyte; calcium influx.

The Ca^{2+}-activated Cl^- channels of ovarian *Xenopus* oocytes were first described by Barish [1], who observed their activation by inducing a Ca^{2+} entry through the endogenous voltage-dependent Ca^{2+} channels [2]. Another way to activate them is by Ca^{2+} mobilization from internal stores [3], either through the activation of membrane receptors coupled to the phosphoinositide cycle [4, 5] or by direct injection of inositol 1,4,5-trisphosphate (InsP3) [6–8].

The role of these channels in the ovarian oocytes is not clear; however, in the stage of ovulated egg, the chloride channels play the important role of generating the fertilization potential that occurs following sperm fusion and which is considered to be responsible of the fast block to polyspermy [9, 10].

These channels have received comparatively little attention per se, as they have been more often used as a tool to monitor cytosolic Ca^{2+} concentration and redistribution [3, 4]. An important example of this application is the investigation of I_{CRAC}, the calcium-release-activated calcium current, which appears to be present in these cells and which, in this preparation, has been studied indirectly through its effects on the Cl^- current.

To use the Ca^{2+}-activated Cl^- current as an indicator of cytosolic Ca^{2+} changes requires that its properties be known in detail and possible contaminations from other currents carefully taken into account. In recent years, it has become apparent that the relation between Cl^- current and Ca^{2+} concentration is not simply linear but that it approximates the derivative of the Ca^{2+} levels [11]. This property is rather peculiar and its biophysical bases are not easily envisaged.

We therefore undertook this work with the aim of investigating in more detail the properties of the Cl^- current in the membrane of ovarian *Xenopus* oocytes.

Materials and methods

Oocytes. Cells were prepared as described by Dascal and Lotan [12]. Ovary fragments were surgically obtained from anesthesized *Xenopus* laevis females and treated with collagenase (Sigma type IA, 1 mg/ml) to remove the follicular cell layer. The oocytes were left to recover overnight in ND 96 supplemented with gentamicin (0.02 mg/ml) and Na-pyruvate (2.5 mM) in an incubator kept at 18 °C.

Electrophysiology and microinjection. The classical two-microelectrode method (Warner Instr. Oocyte Clamp) was used to voltage-clamp single oocytes. Experimental protocols and data acquisition and analysis were done using the pClamp 6.02 software (Axon Instr.).

The non-metabolizable analogue of $InsP_3$, D-3-deoxy-3-fluoro-myo-inositol 1,4,5-trisphosphate (3-F-$InsP_3$, Calbiochem) was used in place of normal $InsP_3$ [13]. To stimulate calcium release, a third pipette (filled with 200 μM 3-F-$InsP_3$) was inserted into the oocyte under voltage-clamp and a volume of 50 nl was injected into the oocyte using an oocyte injector (Drummond).

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Table 1. Composition of external solutions.

	ND 96	0 Ca^{2+}	Ba^{2+}	0 Na^{+}	Low Cl^{-}
NaCl	96	100	96	-	-
KCl	2	2	2	-	-
$MgCl_2$	1	1	1	1	1
Hepes	5	5	5	5	5
$CaCl_2$	1.8	-	-	1.8	-
$BaCl_2$	-	-	1.8	-	-
EGTA	-	1	-	-	-
TMA-Cl	-	-	-	98	-
Na-gluconate	-	-	-	-	96
K-gluconate	-	-	-	-	2
Hemi-Ca-gluconate	-	-	-	-	3.6

All concentrations are in mM. The solution with $[Cl^-]_o = 15$ mM was obtained by appropriately mixing ND 96 with the 'low Cl^-' solution. EGTA = ethylene glycol-O,O′-bis(2-aminoethyl)-N,N,N′,N′-tetra-acetic acid, TMA = tetramethylammonium.

Solutions. The bath solutions used are listed in table 1. ND-96 was used as control solution. The pH of all solutions was adjusted to 7.6.

Results

Early and late currents induced by 3-F-$InsP_3$ injections. When 3-F-$InsP_3$ was injected into the oocyte under voltage-clamp, the membrane current exhibited a rapid transient followed by a long-lasting, substantially constant phase [4, 7]. Both the early transient and the late sustained current are interpreted as carried by Cl^-, and are thought to monitor, respectively, the initial massive release of Ca^{2+} from $InsP_3$-dependent stores and a Ca^{2+} influx activated by depletion of these stores, i.e. I_{CRAC} [14, 15]. As shown in figure 1, this is confirmed by the fact that the reversal potential (E_{rev}) of the early and late phase occurred close to the Cl^- equilibrium potential of these cells, estimated to be around −20 mV when they are bathed in ND96 [1, 16, 17].

The existence of a calcium influx following store discharge has not been demonstrated directly in these cells, but it can be inferred by removing Ca^{2+} from the external solution during the sustained phase. Figure 1C shows that removal of external Ca^{2+} caused a reduction of the sustained current. For comparison, panel D of figure 1 shows the effects of directly injecting Ca^{2+} ions into the oocyte: the initial transient of Ca^{2+}-activated Cl^- current was not followed by a definite sustained phase and, more importantly, removal of external Ca^{2+} in this case did not lead to a reduction of the current but, on the contrary, to its increase, due to increased leakage through the plasma membrane.

As shown by Parker and Yao [11], the relationship between cytosolic Ca^{2+} and Cl^- current is however not linear. These authors suggested a differential relation-

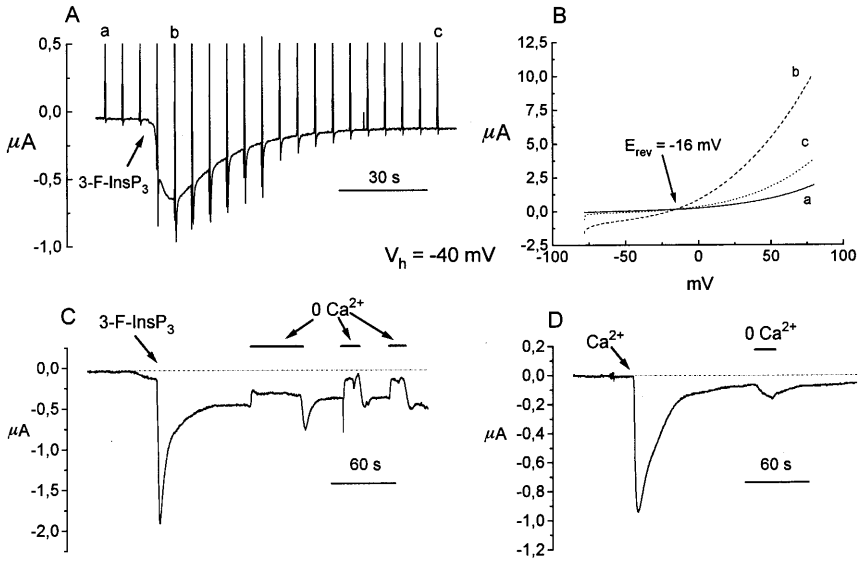

Figure 1. Ca^{2+}-activated Cl^- current induced by 3-F-$InsP_3$ injections in *Xenopus* oocytes. (*A*) 10 nmoles of 3-F-$InsP_3$ were injected at the arrow under voltage-clamp at $V_h = -40$ mV. Voltage ramps from −80 to +80 mV were applied every 6 s to obtain the time course of the I/V characteristic. (*B*) I/V curves from A at rest (a), at the peak of the fast response (b) and during the sustained phase (c), showing that E_{rev} remains at about −16 mV. (*C*) The sustained phase depends on external Ca^{2+}; after 3-F-$InsP_3$ injection, removal of Ca^{2+} from the bath leads to a reduction of the sustained inward current. (*D*) Conversely, when Ca^{2+} was directly injected (1 nmol/oocyte $CaCl_2$), removal of external Ca^{2+} caused an increase in inward current.

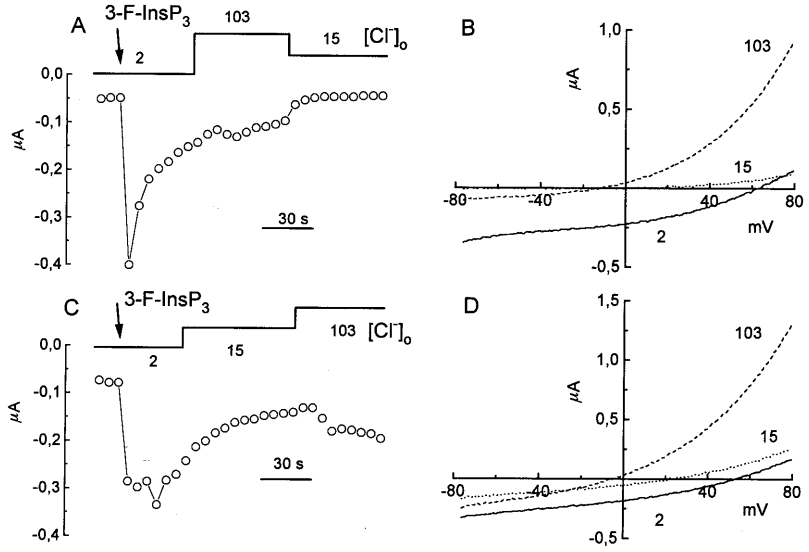

Figure 2. Changes in external Cl^- produce anomalous effects on the sustained current. In (*A*) a reduction from 103 to 15 mM induced a decrease in inward current, while in (*C*) the increase from 15 to 103 mM produced the opposite effect. On the other hand, the corresponding I/V curves, obtained with voltage ramps from −80 to +80 mV applied every 6 s from $V_h = -40$ mV and shown in (*B*) and (*D*), display an E_{rev} that shifts correctly with the changes in $[Cl^-]_o$.

ship between these two quantities, and indeed a behavior in agreement with this hypothesis is visible in figure 1C, where a clear inward transient can be seen upon readdition of Ca^{2+} to the external solution.

Anomalous effect of external Cl^- on conductance. Changes in the external Cl^- concentration during the sustained phase had unexpected effects on the Ca^{2+}-activated Cl^- current, as shown in figure 2. In panel A, 3-F-$InsP_3$ was injected while the oocyte was in low $[Cl^-]_o$ (2 mM, substituted with gluconate). $[Cl^-]_o$ was subsequently increased to 103 mM (control value) and then reduced to 15 mM during the sustained phase. It can be seen that this reduction of the external Cl^- level induced a decrease in the inward Cl^- current, in contrast with the raised electrochemical gradient. In another cell (fig. 2C) $[Cl^-]_o$ was progressively increased from 2 to 15 and to 103 mM; the change from 15 to 103 mM during the sustained phase was associated with an increase in inward current, in spite of the reduced gradient. On the other hand, the current reversal potential shifted in agreement with the Cl^- equilibrium potential, as shown in panels B and D of figure 2, where

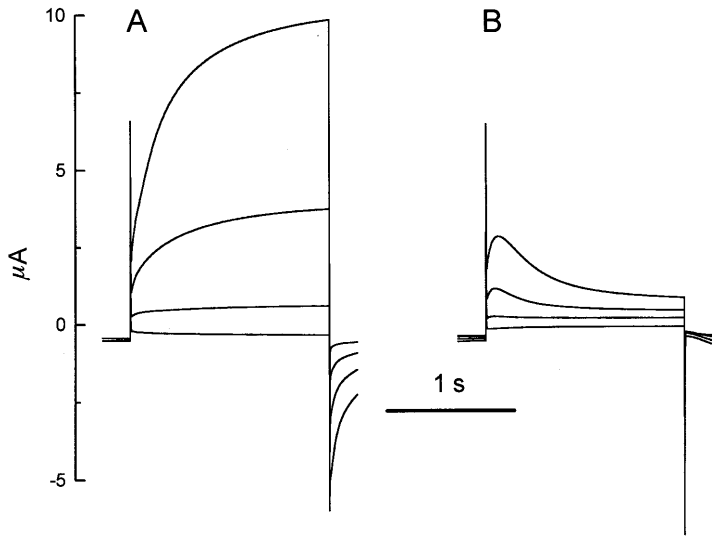

Figure 3. Voltage-clamp pulses from $V_h = -70$ mV to −40, −10, +20 and +50 mV produce currents with different kinetics if they are applied 10 s (*A*) or 300 s (*B*) after the 3-F-$InsP_3$ injection. The large inward tails in (*A*) are replaced by a slowly developing inward 'hump' (whose beginning is just visible at the very end of the traces in (*B*)).

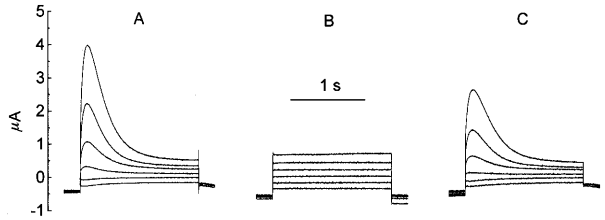

Figure 4. Removal of external Ca^{2+} abolishes the voltage- and time-dependent current during the sustained phase. (*A*) Typical inactivating currents induced by depolarization in control solution (ND96), (*B*) zero external Ca^{2+}, (*C*) recovery in ND96. Voltage pulses were from $V_h = -70$ mV up to $+50$ mV to in 20 mV steps.

the currents in response to voltage ramps corresponding to the various phases are plotted.

Voltage and time dependence of the 3-F-InsP_3-induced currents. To understand better the behavior of the Ca^{2+}-activated Cl^- channels we have studied their voltage and time dependence during the transient and sustained phases after 3-F-InsP_3 injections. In this series of experiments the holding potential was kept at -70 mV and depolarizing pulses to various voltages were applied. During the early transient phase (fig. 3A), this protocol elicited voltage- and time-dependent currents that showed a monotonical rise to a steady level. This kind of kinetics changes gradually to an inactivating behavior at times corresponding to the sustained phase of Cl^- current (fig. 3B). In addition, the large tail currents upon returning to the holding level, seen in panel A, disappear in the traces of panel B. Conversely, during the sustained phase, an inwardly directed ''hump'' appears upon repolarization, corresponding to the current observed by Parekh [5]. All these features are dependent on external Ca^{2+}, as can be observed in the experiment described in figure 4, where the removal of Ca^{2+} reversibly abolished the voltage- and time-dependent components, as well as the repolarization-induced inward hump. The same result could be observed when Ca^{2+} was substituted with Ba^{2+} in the external solution (not shown).

An inward Na^+ current contributes to the current during the late phase. We have investigated in more detail whether the decline of the current was due to inactivation of the Cl^- channels by estimating the slope conductance of the membrane; this was done, as illustrated in figure 5, by applying brief and small ($\Delta V = 10$ mV) steps at the beginning and toward the end of the depolarizing pulse. The ratio $\Delta I/\Delta V$ obtained from these small steps approximates the slope conductance (G_s) of the membrane, which is expected to change as the total current if the channels are inactivating. This analysis was applied during the transient and sustained phase.

Figure 5 shows that there was good proportionality between current amplitude and conductance during the transient phase (panel A), but the proportionality did not hold during the sustained phase, as the conductance remained rather high even at the end of the pulse when the membrane current was strongly reduced (panel B). The Gs/I values at the beginning and at the end of the depolarization remained very similar for the noninactivating currents (mean ratio $= 0.98 \pm 0.04$ SEM, $n = 5$), whilst, in the inactivating currents, Gs/I at the beginning was smaller than at the end of the pulse (mean ratio $= 0.52 \pm 0.11$ SEM, $n = 6$).

This result indicates the presence of a second type of current which, flowing in the inward direction, produces an apparently inactivating behavior. Indeed, *Xenopus* oocytes possess a peculiar kind of Na^+ channel that is slowly inducible by depolarizations [18].

The presence of this current could be revealed using the protocol illustrated in figure 6: from a holding potential of -80 mV, firstly a voltage ramp to $+70$ mV was applied; then a step to $+70$ mV was given and from this

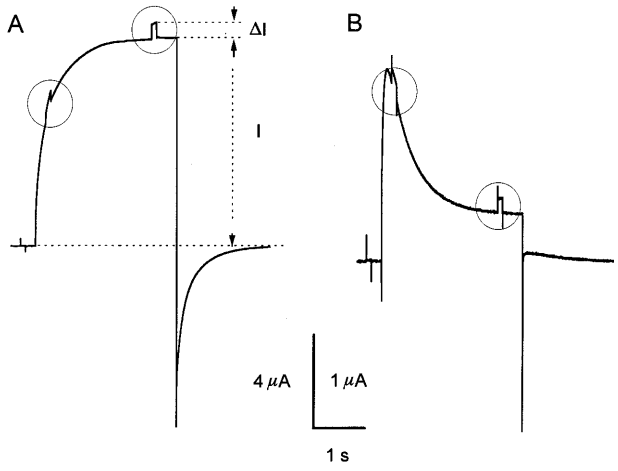

Figure 5. The membrane conductance was estimated by superimposing small steps (10 mV, 100 ms, circled) to a depolarizing pulse to $+70$ mV. The slope conductance G_s was calculated as $\Delta I/\Delta V$. In (*A*) the ratio G_s/I were 6.5 and 7.1 S/A for the first and second pulse respectively (ratio $= 0.92$); in (*B*) they were 10 and 50 S/A (ratio 0.20).

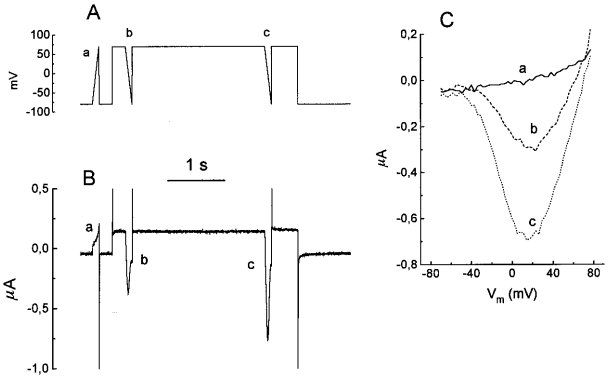

Figure 6. Depolarization-induced Na^+ current. (*A*) Voltage protocol showing the three ramps (a, b, c). (*B*) Membrane current elicited by the voltage command shown in (*A*), in an oocyte in resting conditions and in control solution, (*C*) I/V plot obtained from the ramps in (*B*), after subtraction of the capacitive current.

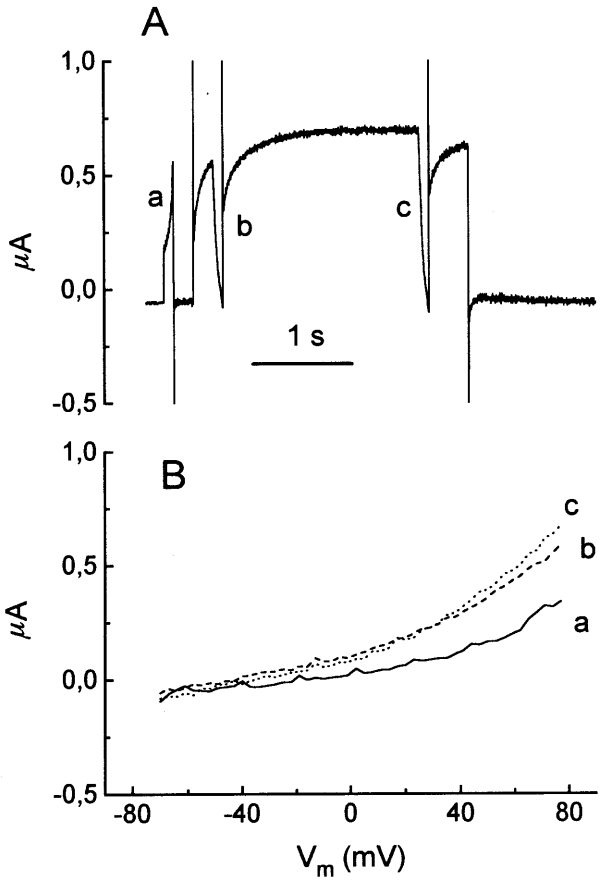

Figure 7. The same voltage protocol as in figure 6A was applied to a resting oocyte bathed in a Na^+-free solution. In this condition no inward current could be observed.

potential two negative-directed ramps to −80 mV were given, one at the beginning and the other at the end of the pulse (fig. 6A).

Figure 6B shows the current in response to such stimulation in a oocyte in resting conditions. Comparing ramps b and c with ramp a it is clear that a new inward current appears that has a reversal potential around +70 mV and that increases from the beginning to the end of the depolarization.

To check that this current is indeed the slowly-inducible Na^+ current described by Baud et al. [18], we repeated the protocol shown in figure 6 in the absence of external Na^+ (replaced by impermeant TMA^+). The results are shown in figure 7 where it is clear that the inward component of the current is not present.

We then investigated the contribution made by this Na^+ current to the time course of the depolarization-activated current after 3-F-$InsP_3$ stimulation by applying the step and ramp protocol of figure 6A. The results of a typical experiment are shown in figure 8: in panel A (illustrating the current recorded 10 s after the 3-F-$InsP_3$ injection) the three ramps (a, b and c) produced I/V curves (panel B) with a progressively more positive reversal potential (−19, 0 and +5 mV). Although slight, these shifts revealed the presence, in addition to the Ca^{2+}-activated Cl^- current, of a current carried by another ionic species with positive equilibrium potential. The contribution of this additional current became evident later (panels C and D), in correspondence with the appearance of the declining time course, about 5 minutes after 3-F-$InsP_3$ injection; in these panels the inward current is conspicuous and the reversal potential of the ramps shifts considerably, −29 mV for ramp a, +45 mV for ramp b, to reach about +80 mV for ramp c. These experiments indicated that the peak outward current was still mainly carried by Cl^-

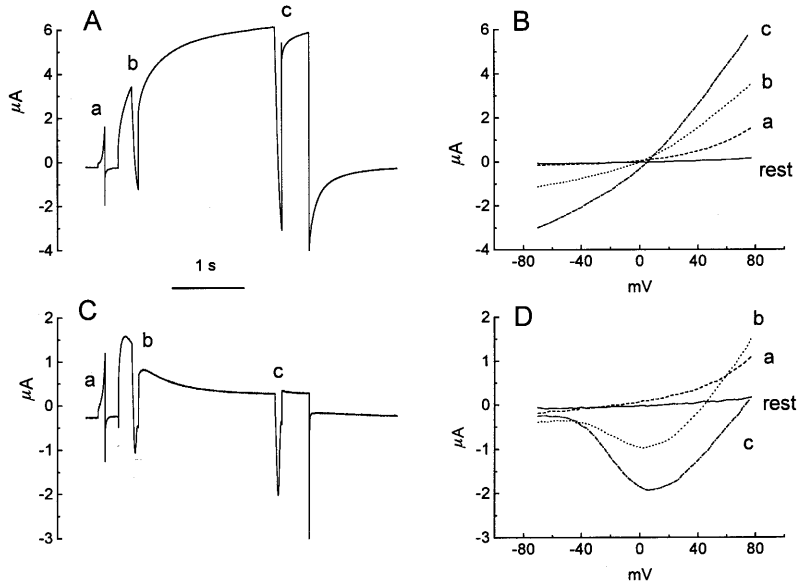

Figure 8. The step and ramps protocol shown in figure 6A was applied to an oocyte 10 s (*A*, *B*) and 300 s (*C*, *D*) after injection of 3-F-$InsP_3$. The ramps a, b, c are plotted together with a control ramp (solid line) obtained in resting conditions before the 3-F-$InsP_3$ injection. The presence of the inward Na^+ current is evident in (*D*) and detectable in (*B*) by the values of the ramp reversal potentials.

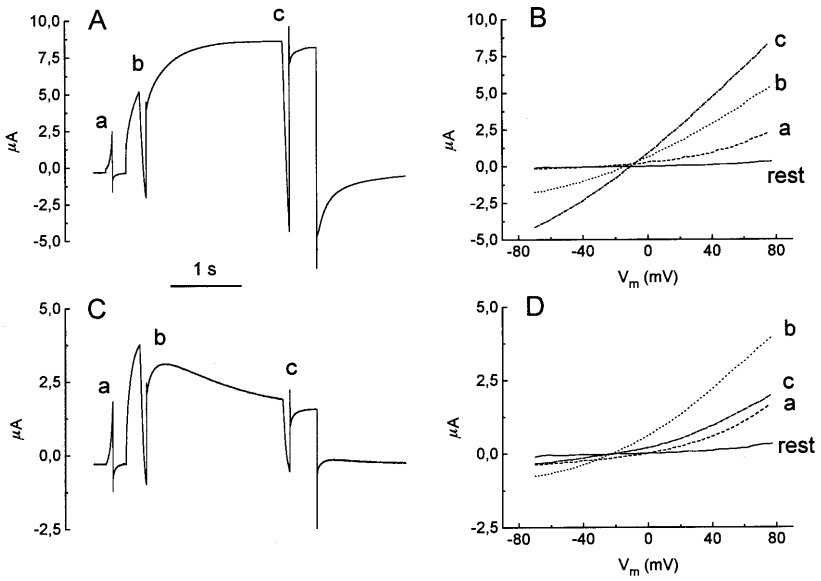

Figure 9. In the absence of external Na^+ neither inward current nor positive shift of the reversal potential can be observed. (*A*, *B*) Step and ramps protocol applied 10 s after 3-F-$InsP_3$ injection, (*C*, *D*) after 300 s from the 3-F-$InsP_3$ injection.

and that this conductance then underwent almost complete inactivation.

The same kind of stimulation applied to an oocyte bathed in zero external Na^+ produced the results observed in figure 9. No significant shifts in the I/V curves obtained by the ramps can be observed, and no inward current is visible in panels C and D, 5 minutes after the 3-F-$InsP_3$ injection, confirming the role of the Na^+ channels.

Discussion

The membrane currents elicited by depolarizations in *Xenopus* oocytes, in which release of stored Ca^{2+} has been induced, exhibit a complex behaviour. Our experiments show that this complexity is due to the presence and interplay of three different currents: i) the store-depletion-activated current I_{CRAC} carried by Ca^{2+}, ii) the Ca^{2+}-activated Cl^- current, and iii) the slowly-inducible, depolarization-activated Na^+ current. Since the direct measurement of I_{CRAC} proved to be elusive and therefore Ca^{2+} changes in the *Xenopus* oocyte are often monitored through the use of the Ca^{2+}-activated Cl^- current, we thought it important to isolate and investigate the properties of this current.

The Ca^{2+}-activated Cl^- current. The results of our experiments show that this current has a number of peculiar features. First of all the anomalous dependence on the Cl^- concentration gradient, illustrated in figure 2: a reduction in external Cl^- reduces Cl^- efflux instead of increasing it, and vice versa. This behaviour is opposite to the expectations from the constant-field equation, and suggests that the presence of external chloride ions is required for channel opening. This kind of property has been described in various K^+ channels [19, 20] and also in Cl^- channels of the ClC family, such as ClC-0, a *Torpedo* chloride channel: in these structures it has been shown that the gating mechanism is affected by the presence of the permeating anion [21, 22].

On the other hand, the $[Cl]_o$ changes are correctly reflected in the E_{rev} shifts, indicating that other ions, such as Ca^{2+} and K^+, contribute negligibly to the overall current.

Another characteristic of this current is the change in kinetics upon depolarizations, between the monotonically rising type during the transient phase to the inactivating type during the sustained phase. We have seen that at high depolarizations an additional current component occurs: the slow induction of a Na^+ current flowing inwardly. Although the inactivating behaviour may be contaminated by the presence of this current, our results suggest that the Cl^- conductance truly declines: this statement is supported by the following observations: i) the current decline is still apparent at membrane voltages close to the Na^+ equilibrium potential, where the contribution from Na^+ current is negligible; ii) the inward tail currents disappear during the sustained phase; iii) current decline is still present (although reduced) when Na^+ is removed from the external solution (fig. 9).

A simple interpretation of this phenomenon might be the following: during the transient phase, Ca^{2+} ions released from internal stores saturate the activation of Cl^- channels which are then opened by depolarization with a monotonical time course. The situation is different during the sustained phase: in this case Ca^{2+} enters from the exterior and its influx depends on the transmembrane electrochemical gradient. At the beginning of the depolarizing step the submembrane Ca^{2+} concentration might be sufficiently high to activate sev-

eral Cl^- channels; however the reduction of the driving force for Ca^{2+} entry and the consequent decrease in submembrane Ca^{2+} level will cause a decline in the number of activated Cl^- channels.

Parekh [5] has recently reported another kind of inactivation, occurring on a slower time scale, which probably has a different origin and which cannot be explained by the above hypothesis.

From our results, it is clear that the characteristics of the Ca^{2+}-activated Cl^- current are very different from those of an ideal Ca^{2+} indicator: voltage and time dependence and sensitivity to external chloride strongly limit its capacity for a quantitative description of the Ca^{2+} levels. All studies using this current to monitor cytosolic Ca^{2+} changes or transmembrane Ca^{2+} movements, such as the CRAC current, must take into account these peculiar properties.

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