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CHLORIDE CURRENT INDUCED BY INJECTION OF CALCIUM INTO *XENOPUS* OOCYTES

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SUMMARY

1. Membrane currents of *Xenopus* oocytes were studied with the membrane under voltage clamp.

2. Intracellular injection of the calcium-chelating agent EGTA reduced, or abolished, the transient outward chloride current normally activated by membrane depolarization.

3. Intracellular injection of calcium ions evoked large membrane currents, which inverted direction close to the chloride equilibrium potential.

4. Injections of strontium, or barium, were less effective than calcium, while magnesium was ineffective.

5. Large chloride currents could be evoked by calcium injections in oocytes which showed only small or no transient outward currents.

6. The current activated by calcium injection increased with increasing depolarization up to high (*ca.* +60 mV) positive potentials, even though the transient outward current was suppressed by strong depolarization.

7. The results indicate that the transient outward current depends upon entry of calcium through voltage-gated calcium ion channels and show that the oocyte membrane contains numerous chloride channels which are activated by intracellular calcium. Only a few of these chloride channels are activated by depolarization.

INTRODUCTION

The surface membrane of fully grown ovarian oocytes of the frog *Xenopus laevis* displays a transient chloride current, which is seen during depolarization of the oocyte membrane and which is dependent upon external calcium (Miledi, 1982; Barish, 1983). We describe here a further study of this current, using intracellular injection of calcium and the calcium-chelating agent EGTA.

METHODS

Experiments were carried out on ovarian oocytes from adult frogs (*Xenopus laevis*), which were usually treated with collagenase to remove follicular and other enveloping cells. Oocytes were incubated in a solution of collagenase (Sigma C-2139; 125–250 u. ml^{-1}) made up in normal Ringer solution and gently agitated for about 1 h at room temperature. The collagenase solution was then replaced by normal Ringer solution, and the bottles containing the oocytes were shaken by hand

until the cell envelopes were seen to detach from the oocytes. Finally, the Ringer solution was replaced by sterile Barth fluid with antibiotics (Gundersen, Miledi & Parker, 1984*a*), and the oocytes were kept in an incubator at 18 °C for use on subsequent days. For electrophysiological study, the oocytes were in a bath continuously perfused with frog Ringer solution (NaCl, 115 mM; KCl, 2 mM; $CaCl_2$, 1·8 mM; HEPES, 5 mM at pH about 7) at room temperature (20–25 °C), and the membrane potential was voltage clamped (Kusano, Miledi & Stinnakre, 1982; Miledi, 1982).

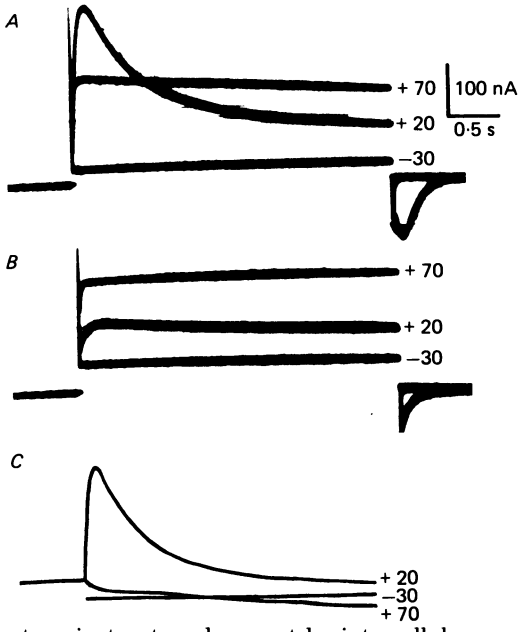

Fig. 1. Abolition of the transient outward current by intracellular pressure injection of EGTA. *A* and *B*, the oocyte was clamped at a holding potential of −100 mV, and the traces show the resulting currents when the potential was stepped to the values indicated (in mV) during a pulse of 3 s duration. In this and other Figures, outward membrane currents correspond to upward deflexions. Temperature 22–24 °C. Records from a normal oocyte bathed in high-calcium (11·8 mM) Ringer, before (*A*) and after (*B*) injection of EGTA. Injection was made with two pressure pulses of 2 bar and 200 ms duration. The total quantity of EGTA injection was roughly 200 pmol (estimated from the size of the fluid droplet expelled when similar pulses were applied with the pipette in the air), and the oocyte volume was *ca.* 1 μl. *C*, difference between the currents recorded before and after EGTA injection, obtained by subtraction of the traces in *A* and *B*.

Intracellular injection of solutions was carried out using a pneumatic pressure system (Stinnakre, 1979), usually with pulses of around 0·4 bar and durations of a few hundred milliseconds. An estimate of the volume injected by each pressure pulse was made by measuring the diameter of the fluid droplet expelled with the pipette tip in the air, prior to penetration. However, pipettes containing calcium solution often blocked after injection of a few pulses, so this calibration is only approximate. For intracellular injection the solutions of divalent cations were prepared using the chloride salts, and contained HEPES set to a pH of about 6·8 with KOH. Except in the case of the calcium solution, 2 mM-EGTA was added, to chelate contaminating calcium. Some early experiments were done using unbuffered calcium chloride solution, which gave essentially similar results to the buffered solution. In experiments to chelate intracellular calcium, the injection solution contained 0·5 M-EGTA (ethylene glycol-bis (β-aminoethyl ether) *N,N'*-tetraacetic acid), set to pH 7 with KOH. Results similar to those described here were seen in experiments in which calcium or EGTA was injected by ionophoresis. Pressure injection was later preferred, because larger amounts could be delivered quickly.

RESULTS

Reduction of the transient outward current by internal EGTA

Depolarization of *Xenopus* oocytes to potentials around 0 mV elicits a transient outward current, which is carried by chloride ions, and which is dependent upon the presence of calcium ions in the bathing solution (Miledi, 1982; Barish, 1983). This

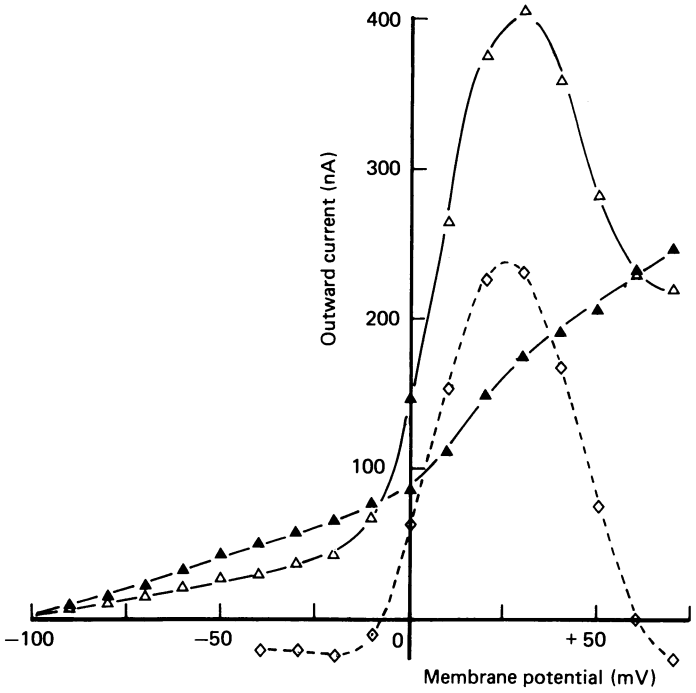

Fig. 2. Current–voltage relationships from the same oocyte as Fig. 1 *A* and *B*, measured before (△) and after (▲) intracellular injection of EGTA. The difference between these two curves is also shown (◇, dashed curve). Currents were measured at the peak of the transient outward current (about 120 ms after the pulse onset), and are expressed relative to the holding current at −100 mV. Intervals of about 20 s were allowed between stimuli. Ringer solution contained 11·8 mM-calcium. Curves were drawn by eye.

current is illustrated in Fig. 1*A*, recorded from an oocyte bathed in high calcium (11·8 mM) Ringer solution in order to increase the amplitude of the response. In this solution, depolarization to −30 mV failed to activate the transient outward current, whilst it was maximal at about +30 mV (Fig. 2), and was suppressed at +70 mV.

Intracellular injection of EGTA greatly reduced the amplitude of the transient outward current (Fig. 1). The response was often reduced to a small fraction within a minute or less of the injection of a single pulse of EGTA, suggesting that EGTA distributes very quickly throughout the volume of the oocyte. A consistent reduction or abolition of the transient outward current was seen in all oocytes examined. Similar results were also obtained using ionophoretic loading of EGTA, although in this case much longer periods of injection (*ca.* 30 min) were required to block the transient outward current.

Fig. 2. shows the current–voltage relationships, measured before and after EGTA injection, from the same oocyte as in Fig. 1. The passive current required for polarization over the range −100 to −20 mV increased slightly after the injection, probably as a result of damage caused by insertion of the EGTA pipette or by the pressure injection itself. However, the striking feature was the almost complete

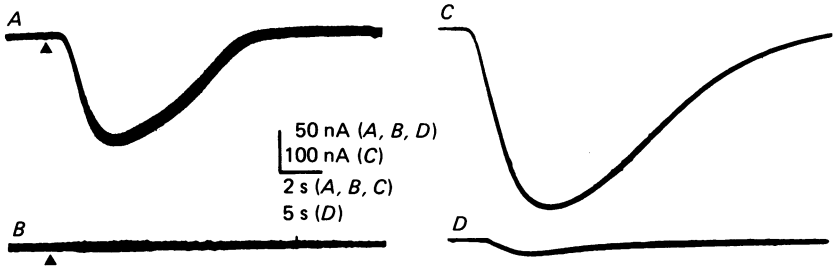

Fig. 3. Currents elicited by intracellular injection of various divalent cations. Records were obtained with oocytes clamped at −60 mV. *A* and *B* were obtained from the same oocyte. Traces show currents in response to injection of *ca.* 0·5 pmol calcium (*A*), 2 nmol magnesium (*B*), 60 pmol strontium (*C*) and 25 pmol barium (*D*). The injection solutions contained 50 mM-$CaCl_2$, 500 mM-$MgCl_2$, 500 mM-$SrCl_2$ or 50 mM-$BaCl_2$, together with HEPES buffer and EGTA to chelate contaminating calcium (see Methods for further details). Amounts of ions injected were estimated as described in the Methods. After obtaining the trace in *B*, the magnesium pipette was withdrawn and it had not become plugged during impalement.

elimination of the outward current activated at potentials between about −10 and +60 mV. This was so in spite of the fact that the external calcium concentration was 11·8 mM and this is known to greatly increase the transient outward current (Miledi, 1982; Barish, 1983).

Intracellular injection of calcium

Pressure injection of calcium chloride into oocytes elicited large, slowly decaying membrane currents (Figs. 3*A*, 4, 5 and 6). These currents were inward when the membrane potential was clamped at −60 mV. In contrast, the transient chloride current seen on depolarizing the membrane is usually outward, because activation of the current normally occurs at potentials more positive than the chloride equilibrium potential. However, in some instances we have seen inward currents in oocytes bathed in normal Ringer solution, presumably because in these oocytes the currents were large, and also perhaps because the chloride equilibrium potential was more positive.

In contrast to injection of calcium, injection of similar or larger volumes of solutions containing EGTA or $MgCl_2$ (Fig. 3*B*) usually gave only small or no changes in holding current. Thus, the responses illustrated almost certainly arose from a specific action of calcium ions, and not from injection of chloride ions or because of damage caused by the fluid injection. The membrane of *Xenopus* oocytes is sensitive to mechanical disturbance and contains channels opened by pressure or stretch. In a few oocytes this was the source of a current during injection; but these 'artifactual'

responses could readily be distinguished from the calcium-activated currents by their short latency, rapid rise and fall, and reversal potential close to 0 mV.

It is known that agents which block calcium channels, such as lanthanum and manganese, also abolish the transient outward current (Miledi, 1982). However, the current induced by intracellular injection of calcium was not blocked by the addition of 10 mM-manganese to the bathing solution, indicating that extracellular calcium is not involved in generating this response.

Most experiments were made on oocytes which had been treated with collagenase to remove follicular and other enveloping cells, but similar responses to calcium injections were obtained from untreated oocytes. Thus, the calcium-activated current arose from the oocyte membrane proper, and was not altered by the presence of the follicular cells.

Injections of calcium into the animal (black) hemisphere of the oocyte usually gave larger responses than similar injections into the vegetal (white) hemisphere, even though there were large variations because of different amounts of calcium ejected by nominally similar pressure pulses. For example, calcium pulses of 0·4 bar and 0·1–0·2 s duration gave a mean current of 20 ± 12 nA (five oocytes; mean $\pm$ S.E. of mean) when the injections were made into the vegetal hemisphere, while similar injections into the animal hemisphere gave a mean current of 260 ± 80 nA (nine oocytes).

Intracellular injection of other divalent cations

Intracellular injections of magnesium ions were almost completely ineffective in eliciting membrane currents. For example, the oocyte in Fig. 3*A* and *B* gave a response to injection of calcium at *ca.* 0·5 pmol, whilst injection of roughly four thousand times this concentration of magnesium elicited no detectable response. Injections of strontium and barium elicited currents (Fig. 3*C* and *D*), but these ions were less effective than calcium.

Reversal potential of the calcium-activated current

The reversal potential of the current activated by intracellular calcium was measured by applying calcium pulses with the oocyte clamped at different membrane potentials (Fig. 4). At voltages close to the potassium equilibrium potential (*ca.* -100 mV: Kusano *et al.* 1982) the current was inward, but it decreased in size as the potential was made more positive and inverted to become an outward current at about -20 mV. The oocyte in Fig. 4 showed spontaneous oscillations in membrane current, which are due to chloride ions (Kusano *et al.* 1982). These inverted direction at about the same potential as the calcium-activation current.

A mean value for the reversal potential of the calcium-activated current, obtained from seven oocytes, was -16 ± 2 mV (mean $\pm$ S.E. of mean). This is close to the chloride equilibrium potential in the *Xenopus* oocyte (Kusano *et al.* 1982; Barish, 1983), indicating that the current is largely carried by chloride ions. The current elicited by injection of strontium also inverted at a similar potential; values of -20 and -18 mV were obtained from two oocytes.

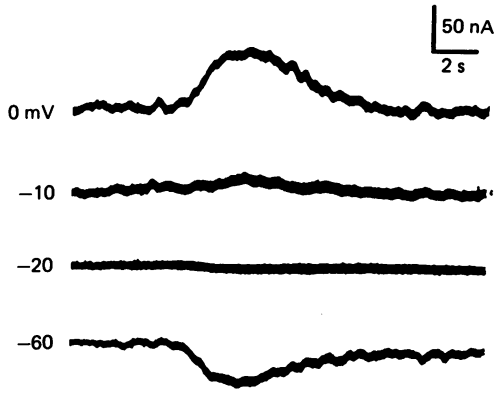

Fig. 4. Reversal potential of the calcium-induced current. Traces show responses to intracellular calcium injections (50 mM-$CaCl_2$ solution; 0·4 bar, 200 ms pulses) applied with the oocyte clamped at the voltages indicated (in mV). Spontaneous oscillations in membrane current were present, which inverted direction at about the same potential as the calcium-activated current.

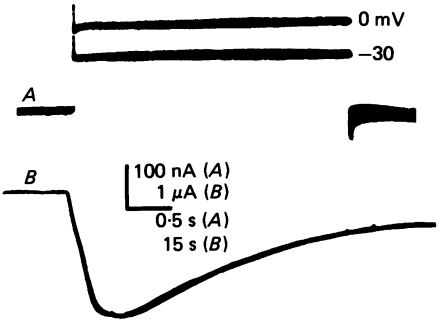

Fig. 5. Large calcium-activated current recorded from an oocyte which showed no transient outward current. *A*, currents elicited by depolarization to −30 mV and 0 mV, from a holding potential of −100 mV. *B*, current elicited by intracellular injection of calcium, with the oocyte clamped at −60 mV. The injection solution contained 50 mM-$CaCl_2$ and 20 mM-HEPES, and was applied using a pressure pulse of 0·4 bar and 5 s duration.

Properties of the calcium-activated current

The time course of the current activated following intracellular calcium injection was very variable, depending in particular on the positioning of the pipette tip and the amount of calcium injected. There was usually a short delay of up to about a second before the onset of the current (Fig. 3*A*). Responses sometimes showed two or more 'humps', suggesting activation of different membrane areas, which presumably contained different densities of chloride channels. Following brief calcium pulses, which activated small responses, the current usually decayed within a few seconds (Fig. 4). However, large injections gave much longer responses (Fig. 5), and in some cases the current did not return to the base line even many minutes after the injection.

The responses to repetitive pulses of calcium, applied at intervals of a few seconds, often showed facilitation, with the current increasing to two or three times its initial size. Whilst this might have arisen because of changes in the amount of calcium ejected from the pipette, a more interesting possibility is that the calcium uptake and buffering capacity of the oocyte became exhausted in the region around the injection pipette.

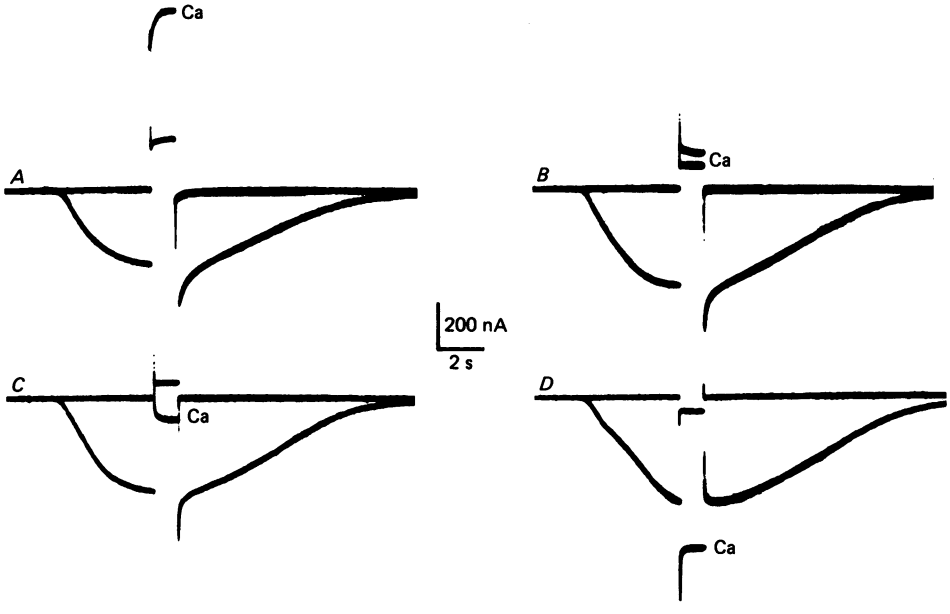

Fig. 6. Currents recorded at different membrane potentials in response to calcium injections. Pulses of calcium were injected with the potential held at −60 mV, and the potential was then stepped briefly to different levels at about the time of the peak response. Intervals of 1 min were allowed between each injection. Each frame shows two superimposed sweeps, with the same potential step applied during the calcium response and with the oocyte at rest. Traces during the calcium responses are marked Ca. Potentials during the pulses were +40 mV (*A*), 0 mV (*B*), −20 mV (*C*) and −100 mV (*D*).

Large currents (several microamperes) could be elicited by injections of calcium, even in oocytes which showed only small calcium-activated transient outward currents on depolarization. For example, the ooctye in Fig. 5 showed no detectable transient outward current when depolarized to 0 mV, even though the transient outward current is about maximally activated at this potential in normal Ringer solution (Miledi, 1982). In contrast, an intracellular calcium pulse gave a current of about 3 μA (at −60 mV).

The chloride current activated by calcium injection had a mean value of $4{\cdot}1 \pm 1{\cdot}5$ μA, obtained from ten oocytes (two donors) clamped at a potential of −60 mV. This is probably a considerable underestimate of the maximum possible current since we avoided using very large injections of calcium, which gave prolonged responses and tended to damage the oocytes. Nevertheless, the size of this current was much greater than the maximal transient outward current recorded from the

same oocytes (mean = 20±8 nA at 0 mV). The currents activated by calcium injection were of similar size, though of opposite polarity, at potentials of −60 and 0 mV (see Fig. 7). Thus, the size of the calcium-activated current at −60 mV may be directly compared with that of the transient outward current at 0 mV.

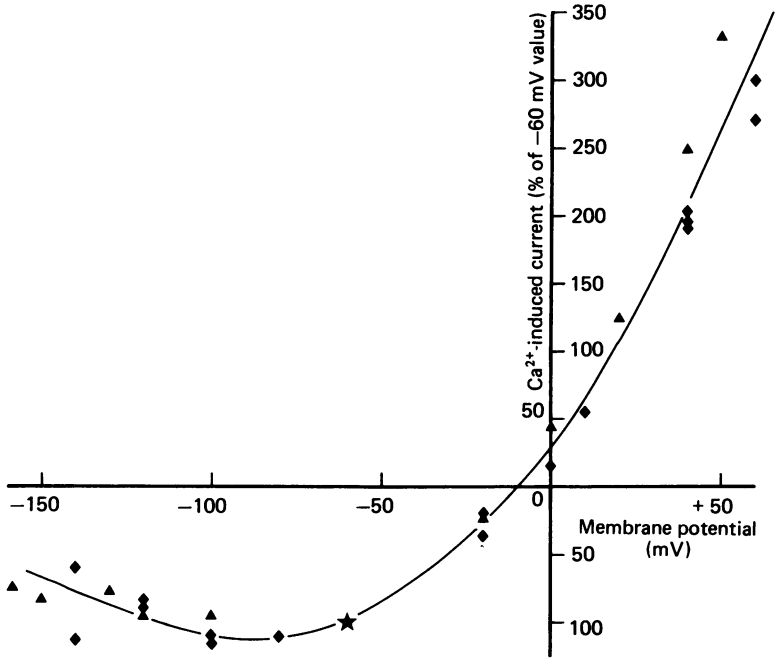

Fig. 7. Current–voltage relationship of the calcium-activated response. Measurements were made from records similar to Fig. 6, and are expressed as a percentage of the response at −60 mV, recorded just before the potential was stepped to a different level. Passive currents, elicited by the potential steps in the absence of calcium have been subtracted. Data from two oocytes (different symbols). Points marked ◆ are from the same oocyte as Fig. 6.

Following injection of large amounts of calcium into the animal (black) hemisphere of the oocyte, the pigment often dispersed around the injection site, forming a white spot. Also, oocytes frequently developed spontaneous fluctuations in membrane current after large injections of calcium, even though the clamp current was stable before the injection. These fluctuations resembled the spontaneous oscillations in chloride current sometimes seen in non-injected oocytes (Kusano *et al.* 1982), and inverted direction at about the chloride equilibrium potential. The current fluctuations persisted for many minutes after the direct calcium-activated current had declined.

Current–voltage relationship of the calcium-activated response

To investigate the voltage dependence of the calcium-activated current, calcium ions were injected while the membrane potential was clamped at −60 mV, and then the potential was stepped to different levels at about the peak of the response (Fig. 6). By this means, the current at each potential could be scaled as a percentage

of the response at −60 mV, so as to compensate for variations in response size with successive injections.

Fig. 7 shows the variation in size of the calcium-activated current with potential. Between about +60 and −50 mV the response was steeply and monotonically graded with potential, but reached a maximal value at about −80 mV and then declined as the membrane was further hyperpolarized.

DISCUSSION

The transient outward chloride current in *Xenopus* oocytes is blocked by removal of external calcium, or by addition of agents presumed to block calcium influx (Miledi, 1982). This suggested that activation of the chloride channels depends upon entry of calcium through voltage-gated calcium ion channels (Miledi, 1982; Barish, 1983), and the present experiments give strong support for this view. Intracellular loading of the oocyte with EGTA reduces or abolishes the transient outward current, presumably because the calcium which enters the cell when the membrane is depolarized is chelated before it can activate the chloride channels. Our experiments also show that intracellular injections of calcium are able to activate a chloride current directly, in the absence of any change in membrane potential.

The chloride current activated by calcium injections increases steeply as the membrane potential is made more positive, up to values of at least +60 mV. This differs markedly from the transient outward current, which (in normal Ringer solution) decreases with polarization beyond about +10 mV, and is almost completely supressed at +40 mV (Miledi, 1982; Barish, 1983). Thus, the supression of the transient outward current at positive potentials appears not to be due to a voltage-dependent closing or decrease in conductance of the calcium-activated chloride channels. Instead, it probably arises because the entry of calcium ions into the oocyte is reduced. One possibility is that calcium entry might be supressed because of a decreased electrochemical driving force at positive potentials. However, a supression potential of +50 mV in normal Ringer solution would imply an intracellular free calcium concentration of 35 μM. A value as high as this seems most unlikely, and preliminary experiments with Arsenazo III (R. Miledi & I. Parker, unpublished observations) indicate a much lower value for the resting free calcium concentration. A more probable explanation is that at positive potentials the voltage-dependent calcium channels either fail to open, or inactivate very shortly after opening.

At negative membrane potentials the calcium-activated chloride current reaches a maximal value at about −80 mV, and then declines with further hyperpolarization. The driving force for chloride efflux is expected to increase as the potential is made more negative, so this behaviour suggests either that the chloride channels show a voltage-dependent closing, or that the single channel conductance decreases with hyperpolarization. A similar rectification at negative potentials is seen with various drug-activated chloride channels in the oocyte, including those activated by native muscarinic receptors (Kusano *et al.* 1982) and by exogenous receptors to serotonin, glutamate and glycine, induced following injection of foreign messenger RNA (Gundersen, Miledi & Parker, 1983, 1984*a*, *b*).

The chloride current activated by large calcium injections decays over a time course of tens or hundreds of seconds, while the decay of the transient outward current is usually complete within one or two seconds. This suggests that the decay of the transient outward current is unlikely to reflect a process intrinsic to the chloride channel, or to the chemical reactions whereby calcium opens the chloride channels. Instead, it seems more likely that the decline of the transient outward current results from inactivation of the voltage-operated calcium channels.

Strontium and barium ions are also able to activate the chloride channels, when applied intracellularly, but magnesium was found to be ineffective. This is in agreement with the experiments of Cross (1981), where intracellular injections of calcium, barium and strontium, but not magnesium, were found to elicit a chloride-dependent activation potential in frog eggs. Contrary to this, however, substitution of barium or strontium for calcium in the bathing solution has been reported to abolish the transient outward current (Barish, 1983). The low efficacy of intracellular strontium and barium, as compared to calcium, may be sufficient to account for this discrepancy, since a reduction in size of the transient outward current to a few percent of its normal size would render it undetectable.

Several other interesting features are also evident in the results. One is that injection of calcium elicited chloride currents much greater than those triggered by depolarization. Thus, the calcium entry occurring during the transient outward current must activate only a very small fraction of the calcium-activatable channels which are present in the normal oocyte membrane. The large currents which can be generated by calcium may, however, play an important role during fertilization, when a potential change occurs due to a chloride flux (Maeno, 1959), which is probably activated by a rise in intracellular free calcium (Cross, 1981). Also, a calcium-dependent chloride current is known to be present in resting ovarian oocytes (Robinson, 1979). This chloride current might arise if the steady leakage of calcium into the cell is sufficient to activate some of the chloride channels in the absence of changes in membrane potential. The direction of this current (from vegetal pole to animal pole) indicates that the resting chloride permeability in the animal hemisphere is greater than at the vegetal end (Robinson, 1979). In agreement with this, our results indicate that larger chloride currents are elicited when calcium is injected into the animal hemisphere, rather than the vegetal.

We have shown previously that the transient outward current is enhanced in oocytes injected with mRNA from rat brain (Gundersen *et al.* 1984*b*). The current in rat brain mRNA injected oocytes has similar properties to that in 'native' oocytes, and is also blocked by internal EGTA (C. B. Gundersen, R. Miledi & I. Parker, unpublished observations). The enhancement of the transient outward current following injection of exogenous mRNA could have come about if translation of the messenger resulted in the incorporation into the oocyte membrane of new calcium channels, or of new chloride channels activatable by calcium. Our results suggest that the formation of new voltage-gated calcium channels is the important factor, since the injection of calcium into normal oocytes shows that the membrane already possesses sufficient chloride channels to be able to account for the enhanced transient outward current in mRNA-injected oocytes.

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