Interaction between injected Ca^{2+} and intracellular Ca^{2+} stores in *Xenopus* oocytes

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Upon two repetitive deep injections of Ca^{2+} into *Xenopus* oocyte (200–300 μm under the membrane), the amplitude of the transient Cl^- current induced by the second injection is several-fold higher than that of the first one. This 'potentiation' persists even at 60–90 min intervals between injections. However, in oocytes permeabilized to Ca^{2+} by the ionophore A23187 in a Ca^{2+}-free solution, the potentiation completely disappears after 30 min. It is proposed that the injected Ca^{2+} is largely taken up by the stores, whereas following the second injection, a higher proportion of Ca^{2+} reaches the membrane, since the stores are already loaded. In ionophore-treated oocytes, the stores lose the accumulated Ca^{2+} over several minutes and are then ready to take up Ca^{2+} again, hindering its arrival at the membrane

Oocyte; Chloride channel; Calcium; Calcium store; *Xenopus laevis*

1. INTRODUCTION

Xenopus oocyte is a widely used model to study mechanisms of Ca^{2+} signalling. Native muscarinic receptors, and other Ca^{2+}-mobilizing receptors implanted in the oocyte's membrane by the injection of exogenous RNA, couple to phospholipase C, and their activation brings about the production of inositol 1,4,5-trisphosphate (Ins-1,4,5-P_3) followed by the release of Ca^{2+} from intracellular stores (see [1] for a review), and also Ca^{2+} influx from the external medium [2]. Elevation of Ca^{2+} levels in *Xenopus* oocytes induces opening of Ca^{2+}-activated Cl^- channels [3–5]. The membrane of the oocyte possesses two Ca-activated Cl^- conductances, fast and slow [6,7]. Intracellular injection of Ca^{2+} (but not Ca^{2+} influx) does not cause inactivation of the Cl^- conductances [7]. Moreover, deep Ca^{2+} injection activates only the slow Cl^- conductance which, unlike the fast one, does not inactivate in a Ca^{2+}-dependent manner [7,8]. The lack of inactivation enabled us to study the interaction of the injected Ca^{2+} with the internal Ca^{2+} stores. Here we report that repetitive deep intracellular injections elicit enhanced Cl^- currents, even when the intervals between the injections are as long as 90 min. However, this effect is largely abolished when the Ca^{2+} stores are depleted by permeabilizing the cell's membranes with the ionophore A23187 in a Ca^{2+}-free solution, suggesting that the potentiation is due to saturation of the Ca^{2+} stores.

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2. EXPERIMENTAL

Xenopus laevis females were anaesthetized and dissected as described [9]. The oocytes were defolliculated by 3–4 h treatment with 1.5 mg/ml collagenase (type 1A; Sigma, Israel) in Ca^{2+}-free frog Ringer (ND96) solution: NaCl 96 mM, KCl 2 mM, $MgCl_2$ 1 mM, Hepes 5 mM (pH 7.6), and stored at 22°C in the same solution with the addition of 1.8 mM $CaCl_2$, 2.5 mM Na-pyruvate, 100 μg/ml streptomycin and 100 units/ml penicillin (see [6] for details).

The currents were recorded in single oocytes (stage 5 or 6, see [10]) using the two-electrode voltage-clamp technique, as described [6,9]. The membrane potential was usually set at −50 mV. Intracellular pressure injection of $CaCl_2$ was done with broken pipettes with tips of 3–6 μm [11]. The injection volume was measured before and after the experiment by injection of the solution into paraffin oil.

Ionophore A23187 was dissolved at 10 mM in dimethyl sulfoxide and stored in light-protected tubes in aliquots of 50 μl at −20°C; final solutions of ionophore in ND96 (0.5–5 μM) were prepared just before use [6].

3. RESULTS

Pressure injection of $CaCl_2$ into the oocyte activates Ca^{2+}-dependent Cl^- current [11,12]. Shallow injections produce only local responses, and Ca^{2+} may not reach all the areas of the cell membrane [8,13]. In our experiments, we always injected 50–200 pmol $CaCl_2$ deep in the cytoplasm, about 200–300 μm under the membrane of the animal hemisphere. Assuming uniform distribution of the injected Ca^{2+} and no extrusion or accumulation by stores (but see below), intracellular free Ca^{2+} concentrations should rise to about 100 μM (and the intracellular Cl^- concentration would rise by 200 μM, which is negligible compared to the resting intracellular Cl^- concentration of about 40 mM [9]). The Cl^- currents elicited by the first injections were in the range 100–800 nA, the time to peak of these currents

was between 20 and 50 s, and decayed over 30–180 s (see Fig. 1). The reversal potential of the current was about -25 mV, as expected for a Cl^- current in these cells [1]. The delay between Ca^{2+} injection and the beginning of Cl^- current was between 20 and 40 s. From here, and assuming a diffusion path of 300 μm, a diffusion coefficient between 5×10^{-6} and 10^{-5} cm^2/s could be calculated, which is 2–3 times lower than in water but several times higher than in squid cytoplasm [14]. This was probably due to the fact that the diameter of the injected drop was 150–200 μm, and thus the diffusion of Ca^{2+} was promoted by the spread of the front of the injected liquid.

Second deep injection of the same amount of Ca^{2+} caused a significantly larger response than the first injection. We termed this phenomenon 'potentiation' (of the Ca^{2+}-induced Cl^- current). When Ca^{2+} was injected in normal Ca^{2+}-containing ND 96 solution at 1–3 min intervals, the current induced by the second injection was 492 ± 95% of that induced by the first injection (Fig. 1A; 11 oocytes, 4 frogs). When Ca^{2+} was injected at 10–15 min intervals, which outlasted by far the duration of the Ca^{2+}-induced Cl^- current itself, the second response was 396 ± 93% of the first one (6 oocytes, 3 frogs; Fig. 1B). Note that in these experiments both injections were done in the same oocyte which was constantly voltage-clamped, and amplitudes of both responses were monitored.

Longer intervals between injections were tested in oocytes of a test group injected with 100 pmol Ca^{2+} and incubated in normal ND 96 for 60–90 min before measuring the response to second injection of the same dose of Ca^{2+}. Intermittently, responses to Ca^{2+} injection were measured in oocytes of a control group not injected previously with Ca^{2+}. The responses were 135 ± 42 nA ($n = 7$) in control and 723 ± 178 nA in the test group (535% of control). The difference between the responses in the two groups was highly significant, $P < 0.001$. Thus, as summarized in Fig. 2 (empty bars), the same potentiation is observed a few minutes and more than one hour after the first injection of Ca^{2+}.

Responses to deep injection of Ca^{2+} were occasionally followed by Cl^- current fluctuations and/or a slow Cl^- current (Fig. 1A and [11]), sometimes even 1 h after the injection. Such currents are rather characteristic of the oocyte [15,16] and may be due to Ca^{2+}-evoked Ca^{2+} release from internal stores [13,17–20]. Thus, it is possible that the injected Ca^{2+} causes saturation of the Ca^{2+} binding sites in the cell, including the available stores, and this leads to elevated free Ca^{2+} levels or to an enhanced Ca^{2+} release from the stores.

To examine the involvement of the stores in potentiation, the stores were partially depleted by a 15 min treatment of the oocytes with the ionophore A23187 in Ca^{2+}-free, EGTA-containing solution [13]. The partial depletion was verified by the disappearance of the first
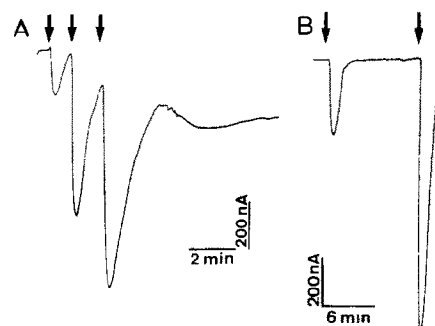
Fig. 1. The effect of repetitive injections (shown by arrows) of about 100 pmol Ca^{2+} into the animal hemisphere of two different oocytes at 1–2 (A) and 15 (B) min intervals. Cl^- currents are inward (correspond to efflux of Cl^- from the cell) and are shown as downward deflections of the current trace. Holding potential was -50 mV in both cases.

phase, and a significant reduction of the second phase, of the response evoked in brain RNA-injected oocytes by serotonin (not shown; see [21]).

In ionophore-treated oocytes, the response to injection of 100 pmol Ca^{2+} was smaller than in control oocytes (114 ± 33 nA, $n = 16$, vs 337 ± 59 nA, $n = 17$, respectively). The ionophore treatment did not eliminate the potentiation: with 1–3 min intervals between injections, the second response was 561 ± 184% of the first (6 oocytes, 2 frogs). Similar potentiation was observed even when the cells were incubated with ionophore for 1–4 h before the first Ca^{2+} injection. However, the potentiation was less pronounced when the interval between the two injections was increased. Thus, with 10–15 min intervals ($n = 5$), the second response was 268 ± 60% of the first one, and with a
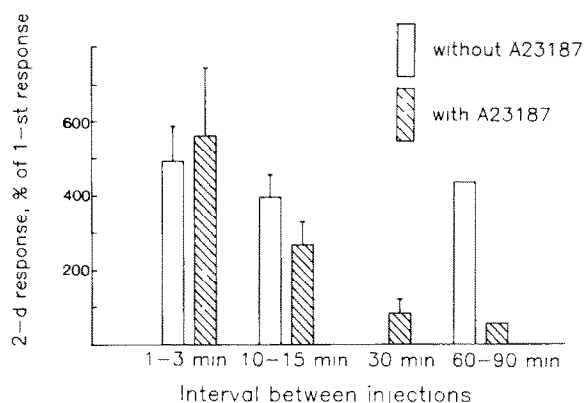
Fig. 2. The dependence of the extent of potentiation on the interval between two consecutive Ca^{2+} injections. The results of experiments in oocytes of 7 frogs are summarized. The dashed bars represent the control experiments done in normal ND 96 solution. The hatched bars represent experiments done on oocytes permeabilized by a 15 min treatment with A23187 and incubated in Ca^{2+}-free, EGTA-containing solution. All bars except the two rightmost show mean ± SE (second response as % of first response), and summarize the experiments in which both first and second injections were measured in the same oocyte. The two rightmost bars represent experiments in which two groups of oocytes were compared. See text for further details.

30 min interval, the second response was not significantly different from the first one ($82 \pm 38\%$, $n = 4$, $P > 0.3$). Longer intervals were tested in an experiment in which two groups of ionophore-treated oocytes were employed. The test oocytes were injected with 100 pmol Ca^{2+} and incubated in the Ca^{2+}-free solution. Control oocytes were incubated in the same way but were not injected with Ca^{2+}. Responses to Ca^{2+} injection tested after 60–90 min of incubation were 236 ± 119 nA ($n = 5$) in the test group and 428 ± 179 ($n = 5$) in control (difference statistically insignificant, $P > 0.2$). An additional injection of Ca^{2+} after 1–3 min displayed the usual potentiation in oocytes of both groups (not shown), suggesting that the Cl^- channels and other components of the cell's response were not damaged. The results of all experiments in ionophore-treated oocytes are summarized in Fig. 2 (hatched bars).

4. DISCUSSION

Here we demonstrate that repetitive deep injections of large doses of Ca^{2+} into the *Xenopus* oocytes evoke progressively larger Cl^- currents, even at very long intervals (more than 1 h), and that the duration of potentiation is dramatically reduced in oocytes permeabilized to Ca^{2+} with the ionophore A23187.

We propose that the mechanism of potentiation involves the loading and possibly even saturation of intracellular Ca^{2+} stores by the injected Ca^{2+}. According to this view, only a small part of the Ca^{2+} ions introduced by the first injection reaches the membrane, a major part being taken up by the stores. A subsequent injection of Ca^{2+} then produces a larger response, because the stores are loaded and can take up much less Ca^{2+}, more of which now reaches the membrane. Importantly, the fact that there is no attenuation in the extent of potentiation even when the injections are separated by up to 90 min intervals suggests that during this time there is no loss of Ca^{2+} from the stores. On the other hand, in oocytes whose membranes are permeabilized to Ca^{2+} with A23187, the stores fully lose the accumulated Ca^{2+} within 30 min, regain their ability to take up Ca^{2+}, and therefore a second injection of Ca^{2+} after 30 min or more does not elicit a larger response. Significantly, the fact that in the ionophore-treated oocytes the potentiation is normal with 1–3 min intervals between injections indicates that the stores' ability to take up Ca^{2+} remains intact. The proposed mechanism also corroborates the finding that, as compared to control oocytes, the amplitude of the response evoked by the first Ca^{2+} injection in ionophore-treated oocytes is smaller: in these oocytes the Ca^{2+} stores are partially depleted, they take up more Ca^{2+}, so less Ca^{2+} reaches the membrane.

There are other possible explanations for our results, but they appear quite unlikely. Thus, the potentiation cannot be explained by activation of an enzyme acting on Cl^- channels, because Ca^{2+} injection has little effect on both fast and slow Cl^- currents evoked by Ca^{2+} influx [7]. Cooperativity at the level of Cl^- channel is possible but cannot explain our results, because when Ca^{2+} is introduced by repetitive *shallow* injections, not reaching the bulk of the cytoplasm, no potentiation is observed [11,12]. Moreover, under such conditions an increase in the amount of injected Ca^{2+} correlates well with an increase in Cl^- current amplitude [11], demonstrating that the latter is a good indicator for changes in the amount of Ca^{2+} arriving at the membrane.

It has been shown that Ca^{2+} injection only slightly [17] or not at all [21] enhances the response evoked by Ca^{2+}-mobilizing neurotransmitters, which act by release of Ca^{2+} from the Ins-1,4,5-P_3-sensitive store. Thus, it appears that the store involved in potentiation phenomenon is the Ins-1,4,5-P_3-insensitive one [18,19], and that the injected Ca^{2+} is taken up by this store. The internal store loading by the injected Ca^{2+} described here provides a new tool for further exploration of the interactions between Ca^{2+}, Ca^{2+} stores and Ca^{2+}-mobilizing transmitters in this highly important model system.

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