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Extracellular calcium participates in responses to acetylcholine in *Xenopus* oocytes

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We tested the contribution of extracellular calcium (Ca_o^{2+}) to membrane electrical responses to acetylcholine (ACh) in native *Xenopus* oocytes. Removal of Ca_o caused a decrease in both the rapid (D_1) and the slow (D_2) chloride currents that comprise the common depolarizing response to ACh in native oocyte. The effect of Ca_o^{2+} removal on the muscarinic response was mimicked by the addition of 1 mM Mn^{2+}, an effective antagonist of calcium influx, though not by antagonists of voltage-sensitive calcium channels. When oocytes were challenged with ACh in Ca^{2+}-free medium, subsequent addition of 1.8 mM $CaCl_2$ resulted in a rapid, often transient, depolarizing current. Similarly to the Ca_o^{2+}-dependent component of membrane electrical responses, the Ca^{2+}-evoked current was reversibly abolished by Mn^{2+}, though not by antigonists of voltage-sensitive calcium channels. Depletion of cellular calcium potentiated the Ca^{2+}-evoked current, implying negative feedback of calcium channels by calcium. Injection of 10–100 fmol of inositol 1,4,5-trisphosphate (IP_3) resulted in a two-component depolarizing current. IP_3 injection promoted the appearance of Ca_o^{2+}-evoked current that was significantly potentiated by previous calcium depletion. We suggest that activation of cell-membrane muscarinic receptors causes opening of apparently voltage-*insensitive* and verapamil or diltiazem-resistant calcium channels. These channels may be activated by IP_3 or its metabolites, which increase following the activation of cell membrane receptors coupled to a phospholipase C. The channels may be identical to receptor-operated channels described in other model systems.

Calcium channel; Inositol phosphate; Chloride current; Muscarinic response; *Xenopus* oocyte

1. INTRODUCTION

Xenopus oocytes serve as an excellent model for studying cell membrane receptors activation, the molecular mechanisms of signal transduction and the mechanisms of activation of ionic channels (see [1] for review). Native oocytes exhibit a complex depolarizing response to ACh [2,3]. The signal transduction pathway proposed for the muscarinic response in oocytes includes activation of a phospholipase C, generation of the two intracellular second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol and mobilization of calcium. The increase in cytosolic calcium concentration activates Cl channels in the oocyte plasma membrane [4–9].

It has been proposed that depolarizing Cl currents in *Xenopus* oocytes result solely from the mobilization of calcium from cellular stores. This hypothesis was based on the apparent independence of the intrinsic muscarinic response [4] of $[Ca_o^{2+}]$. On the other hand, chelation of intracellular calcium [4,10] or its depletion by treatment with the divalent cation ionophore A23187 in Ca^{2+}-free medium [9] or by repeated exposures to the agonist in Ca^{2+}-free medium [4] completely abolished ACh-evoked responses. Dascal et al. [11], however, reported that Ca_o withdrawal reduced the amplitudes of all muscarinic receptors, though calcium currents were not observed (i.e. there was no significant calcium influx during the muscarinic stimulation).

Recently, Snyder et al. [12] reported that the slow component of IP_3-evoked Cl current in *Xenopus* oocytes exhibited marked dependence on Ca_o concentration and could be significantly inhibited by Mn^{2+}, an antagonist of calcium influx. The goal of the present investigation was to re-examine the role of Ca_o in receptor-mediated generation of Cl currents in *Xenopus* oocytes.

2. MATERIALS AND METHODS

2.1. *Experimental animals*

Adult *Xenopus* females, purchased from South African Snake Farm (Fisch Hoek), were maintained at 19–21°C in a 12/12 h light/dark cycle and fed diced beef liver twice weekly. The animals were cold-anaesthetized and ovary fragments were dissected into ND96 medium. Stage 5 or 6 follicle-enclosed oocytes were manually separated from ovary fragments and maintained at 20°C in ND96. When oocytes of the same donors were assayed repeatedly, dissections were spaced 2–3 weeks apart, to allow for a full recovery of the animals from the surgical procedure.

2.2. *Electrophysiology*

The electrophysiological methods were described in detail elsewhere [9,13,14]. Briefly, all experiments were performed in a 0.3 ml perfusion bath under two-electrode voltage-clamp using Dagan 8500 intracellular clamp amplifier. Oocytes were routinely

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clamped at −100 mV to avoid interference by potassium currents. Drugs were added rapidly and directly to the bath in a relatively large volume (>1 ml) in order to avoid the dead time of the perfusion system and the gradual build-up of the agonist concentration in the bath. Changes in $CaCl_2$ concentrations in the medium were effected also by direct, rapid addition of appropriate solutions to the bath.

2.3. *Intracellular IP_3 injections*

Intracellular pressure injections were performed using a third, manually broken micropipette, back-filled with 0.01–1.0 mM IP_3 solution, essentially as described previously [5,9,14,15].

2.4. *Analysis of results*

All experiments were repeated several times in oocytes from at least two different frogs. The number of oocytes assayed for each condition is denoted by n and the number of different donors by N. Experiments were performed by assaying several oocytes within each experiment and mean ± SE values were determined. Statistical significance was determined by the unpaired Student's t-test.

2.5. *Solutions and chemicals*

The composition of ND96 was (in mM): NaCl 96, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1.8, Na-Hepes 5, pH 7.5. Changes in Cl concentrations due to the inclusion or omission of 1.8 mM $CaCl_2$ or 1 mM $MnCl_2$ in the medium brought about only very small changes in the electrode potential and were not, therefore, compensated by corresponding changes in the concentration of NaCl. ACh and IP_3 were purchased from Sigma; $^{45}CaCl_2$ from Amersham. All other chemicals were of analytical grade.

3. RESULTS

3.1. *The dependence of membrane electrical responses on Ca_o*

We assayed the contribution of $[Ca_o]$ to the generation of the common depolarizing Cl responses in *Xenopus* oocytes. The role of $[Ca_o]$ was tested in two protocols: (a) comparison of responses evoked in Ca-replete ND96 medium ($[Ca_o]$ = 1.8 mM) to those evoked in Ca-free ND96 (+0.1 mM EGTA); (b) comparison of responses evoked in Ca-replete ND96 medium to those evoked in ND96 that included 1 mM of $MnCl_2$, an antagonist of calcium influx.

The removal of Ca_o caused a significant decrease of both the rapid (D_1) and the slow (D_2) components of the depolarizing response to ACh (10 μM). In the absence of Ca_o, the amplitude of the rapid component was 74 ± 8% and that of the slow component 62 ± 7%, when compared to the control responses obtained in oocytes of the same donors in Ca-replete medium (n_c = 64, $n_{Ca=0}$ = 81, N = 7; fig.1A). The withdrawal of Ca_o was also mimicked by inhibition of calcium influx into the oocyte. Addition of 1 mM of $MnCl_2$ to ND96 Ca-replete medium resulted predominantly in a reversible decrease of D_2 (fig.1B). The inhibition caused by $MnCl_2$ was larger than that caused by calcium withdrawal (table 1).

These results implied that calcium influx contributes to the amplitude of both components of the muscarinic response, but in particular to the slow, prolonged component (D_2). In many tissues the slow phase of the physiological response is often attributed to calcium influx via voltage-sensitive calcium channels (VSCs).

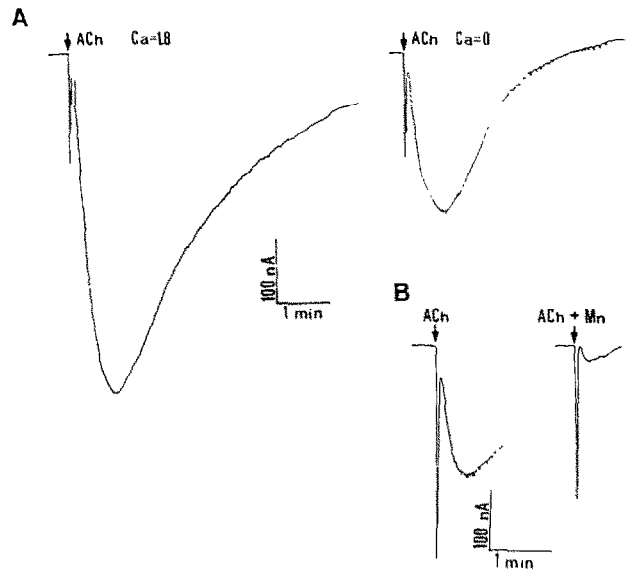

Fig.1. The effect of calcium withdrawal and $MnCl_2$ on responses to ACh. Two oocytes of the same donor were challenged with 0.1 mM ACh in ND96 Ca-replete medium (A, left panel) or in Ca-free ND96 (+0.1 mM EGTA) (A, right panel). Two oocytes of the same donor were challenged with 0.1 mM ACh in Ca-replete ND96 medium (B, left panel) or in the presence of 1 mM of $MnCl_2$ (B, right panel). $MnCl_2$ was added 1 min before ACh. The holding potential in both experiments was −100 mV. Solid arrows indicate the addition of ACh.

Although the involvement of VSCs was unlikely at the experimental holding potential (−100 mV), we have tested the effects of specific antagonists of VSCs, verapamil (1 μM) and diltiazem (10 μM). Verapamil and diltiazem had a moderate inhibitory effect on the amplitude of the rapid component of the response to ACh (D_1). This effect was most likely non-specific, since it was observed both in the presence and the absence of extracellular calcium. They had no effect on the slow component (D_2) of the response (not shown).

Table 1

The effects of Ca_o or $MnCl_2$ on ACh response and Ca_o-evoked current

Response	Relative response (% of control)			
	Ca^{2+} = 1.8	Ca^{2+} = 0	Ca^{2+} = 1.8, Mn^{2+} = 1	n, N
D_1	100	74 ± 8*	–	81, 7
D_2	100	62 ± 7*	–	62, 7
D_1	100	–	74 ± 23	19, 4
D_2	100	–	39 ± 10*	18, 4
Ca_o-evoked current	100	–	18 ± 10*	13, 3

Xenopus oocytes were challenged with 0.1 mM ACh and the magnitudes of rapid (D_1) and prolonged (D_2) depolarizing currents determined in normal ND96 solution ([Ca] = 1.8 mM), in Ca-free ND96 and in normal ND96 in the presence of 1 mM $MnCl_2$. The last row describes the effect of $MnCl_2$ on Ca_o-evoked chloride current in ACh-stimulated oocytes

3.2. *Receptor-mediated calcium influx into oocytes*

The results of the above-described experiments suggested that receptor activation causes an increased influx of calcium into the oocyte. To test this hypothesis, we have conducted electrophysiological experiments in voltage-clamped oocytes according to the following protocol. Oocytes were challenged with ACh in Ca-free ND96 medium. At various times after the beginning of exposure to the agonist, the medium was rapidly changed to Ca-replete ND96 that included the same concentration of ACh. In most oocytes, the change to a Ca-replete medium resulted in a generation of additional depolarizing current. In many oocytes, however, the chelation of calcium resulted in a continuously increasing depolarizing current (this phenomenon was described as 'deterioration' by Dascal et al. [4]) and a subsequent addition of calcium resulted in a hyperpolarizing current. Cells that exhibited this behaviour were discarded.

When measured 1–2 min after the addition of 10 μM ACh, this Ca-evoked current was usually prolonged and relatively modest (mean amplitude 60 ± 18 nA, n = 28, N = 5) and required continued presence of ACh, as the removal of the agonist resulted in a rapid disappearance of the current (fig.2A). In some experiments, the Ca_o-evoked current was transient, despite continuous presence of the agonist (fig.2B). The calcium-evoked current could be elicited within 30 s of the application of the agonist and did not increase upon prolonged exposure to ACh.

The Ca-evoked currents in ACh-stimulated oocytes were reversibly antagonized by 1 mM Mn^{2+} (table 1), though not by the calcium channel antagonists.

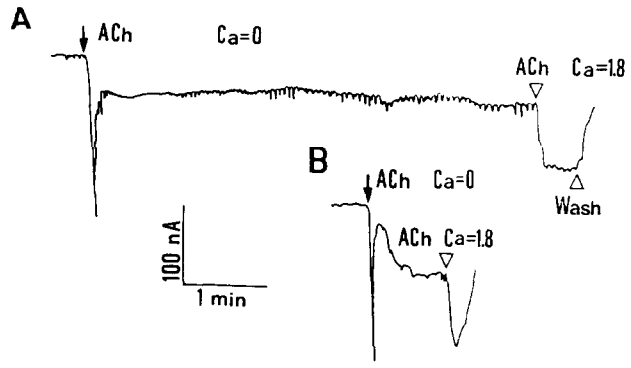

Fig.2. ACh-induced Ca_o-evoked current. Two representative tracings of Ca_o-evoked depolarizing current in oocytes challenged with ACh. (A) An oocyte was challenged with 0.1 mM ACh (solid arrow) in calcium-free ND96. The addition of 1.8 mM Ca approximately 5 min after the beginning of the exposure to ACh (open down-pointing arrow), resulted in a prolonged depolarizing current that rapidly disappeared upon removal of ACh (open up-pointing arrow). (B) Similar response showing a transient Ca_o-evoked current. Calcium (1.8 mM) was added approximately 1 min after the beginning of the exposure to ACh. Holding potential was −100 mV in both experiments.

3.3. *Depletion of calcium potentiates Ca_o-evoked current*

The magnitude of the Ca_o-evoked current in the presence of ACh was affected by the previous treatment of the oocyte. Depletion of oocyte calcium potentiated the subsequent response to the addition of $CaCl_2$. In a typical experiment (see fig.3A), control oocytes were challenged with 10 μM ACh in Ca-replete ND96, washed free of ACh with Ca-replete ND96 for 4 min (to allow for recovery of calcium released by ACh) and additionally with Ca-free ND96 for 1 min. The oocytes were then challenged again with ACh in Ca-free ND96. After 1 min, the concentration of calcium was restored to 1.8 mM. The resulting Ca_o-evoked current was 57 ± 12 nA (n = 9, N = 2). Oocytes of the same frog were then exposed to a different protocol. They were challenged by 10 μM ACh in Ca-free ND96, washed free of ACh in Ca-free medium for 5 min (to deplete oocyte calcium) and challenged again with ACh in Ca-free ND96 for 1 min. The change to Ca-replete ND96 (with ACh) resulted in a Ca_o-evoked current that was 162 ± 48 nA (fig.3B, n = 7, N = 2). Hence, previous depletion of calcium enhanced the Ca_o-evoked current by 284%.

3.4. *IP_3 may serve as a second messenger for calcium influx*

It was recently reported [12] that the slow, prolonged

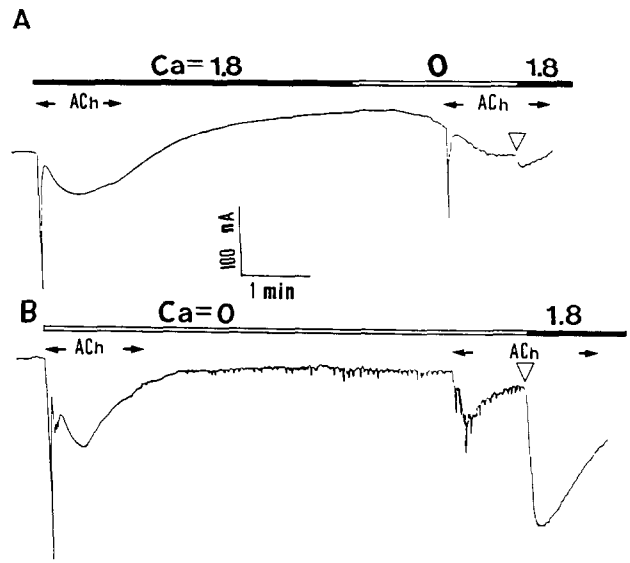

Fig.3. The effect of calcium depletion on ACh-induced, Ca_o-evoked current. Two oocytes of the same donor were tested for the effect of calcium depletion on Ca_o-evoked current. In panel A, an oocyte was challenged with 0.1 mM ACh in calcium-replete medium for 1 min. Following 4 min of wash with calcium-replete medium, calcium was removed for an additional minute and the cell then challenged again with ACh in Ca-free medium. After 1 min, calcium was added to a final concentration of 1.8 mM (open arrow). In panel B, the oocyte was challenged with ACh in calcium-free medium for 1 min and maintained in calcium-free medium until the second challenge with ACh. Calcium (1.8 mM, open arrow) was added 1 min after the second exposure to ACh. The holding potential was −100 mV.

depolarizing current evoked in *Xenopus* oocytes by injection of various isomers of IP_3 is sensitive to Ca_o. The authors of that study used very high concentrations of IP_3 isomers and very high extracellular Ca^{2+} (6 mM). We have, therefore, repeated some of the experiments using lower range of IP_3 concentrations. Injection of 10–100 fmol of IP_3 resulted in a two-component depolarizing current. At these concentrations of IP_3, the rapid current was often fused with the slow current (fig.4A). In Ca-free medium, however, the slow component of this current was often late in appearance and prominent fluctuations were observed (fig.4B). Subsequent change to a medium containing 1.8 mM $CaCl_2$ produced a rapid onset of a transient depolarizing current that was dose-dependent on the amount of injected IP_3. This was inhibited by 90 ± 3% by 1 mM $MnCl_2$ (n = 9, N = 4; not shown). Similarly to the ACh-evoked Ca_o-dependent current, previous depletion of cell calcium by two consecutive responses in Ca-free medium resulted in a marked potentiation (by 400%) of the Ca_o-dependent current (fig.4A,B). As a rule, IP_3-dependent Ca_o-evoked currents were transient and much more sharp than similar currents in oocytes challenged with ACh (see figs 2,3).

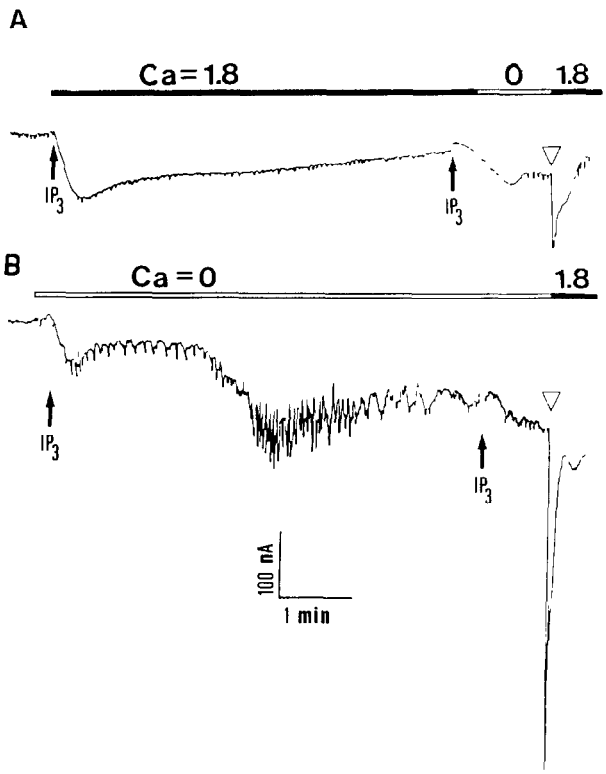

Fig.4. The effect of calcium depletion on IP_3-induced, Ca_o-evoked current. Two oocytes of the same donor were tested for the effect of calcium depletion on Ca_o-evoked current. In panel A, an oocyte was injected with 10 fmol IP_3 (solid arrow) in calcium-replete medium. After 6 min the cell was re-injected with the same amount of IP_3 (solid arrow) and the medium changed to Ca-free ND96 for 1 min. The cell was then challenged again with Ca-replete medium (open arrow). In fig.3B, the protocol was identical, except that the oocyte was maintained all the time in calcium-free ND96. The holding potential was −80 mV.

4. DISCUSSION

Until recently, it was generally accepted that depolarizing membrane electrical responses to stimulation of cell membrane receptors in *Xenopus* oocytes proceed mainly via mobilization of calcium from cellular stores by IP_3. This hypothesis was based on the persistence of responses in calcium-free medium, their gradual disappearance when calcium stores are depleted by repetitive challenges with an agonist in calcium-free medium and their subsequent restoration when cell calcium was recovered [4]. This mechanism was further validated by reports that agonists or microinjected IP_3 evoke rapid efflux of $^{45}Ca^{2+}$ [4,8] and direct rise in cellular free calcium monitored by aqueorin [7] or Fura-2 [16]. Indeed, we have reported similar results for the rapid acquired response to thyrotropin-releasing hormone in oocytes injected with pituitary tumor GH_3 cell mRNA [13].

Dascal et al. [4,11] have reported that responses to ACh are blunted in Ca-free medium and partially inhibited by Mn^{2+}. Their protocol, however, included high concentration of Mn^{2+} (18 mM) and prolonged pre-incubation periods. Under these conditions, it is possible to deplete oocyte calcium. We have used much lower concentrations of Mn^{2+} (1 mM) and short incubation times (<2 min). Our results unequivocally show that blockade of calcium entry causes a significant inhibition of both D_1 and D_2 components of the ACh response. These findings confirm the reports of Dascal et al. [4,11].

Irvine et al. [17] have proposed that a combination of IP_3 and inositol 1,3,4,5-tetrakisphosphate (IP_4) mobilizes calcium from the medium in sea urchin oocytes. Subsequently, Parker et al. [18] have shown that IP_4 opens calcium channels in oocytes and that this action of IP_4 is more pronounced in oocytes voltage-clamped around −100 mV and is assisted by co-injection of IP_3. Snyder et al. [12] have demonstrated that the slow component of the IP_3 response requires extracellular calcium and is inhibited by Mn^{2+}. They have also reported that the increased calcium influx could be evoked by a number of IP_3 isomers, though not by IP_4. Hence, it appears that IP_3 alone, its metabolites or combination thereof, may mediate the opening of calcium channels in the membrane of sea urchin and *Xenopus* oocytes. Kuno and Gardner [19] have demonstrated direct opening by IP_3 of calcium channels in excised patches from human T-lymphocytes.

Unlike Snyder et al. [12], who injected supramaximal amounts of IP_3, we have observed that the injection of IP_3 in Ca-free medium resulted in a delay in the appearance of the slow component of the response and

pronounced fluctuations, rather than its complete inhibition. This may be related to the much smaller amounts of injected IP_3. On the other hand, it is possible that in Ca-replete medium the continuous influx of calcium affects the opening of chloride channels or depresses calcium mobilization from cellular stores and, consequently, reduces the fluctuations characteristic of the response to IP_3 (see below).

The proposed mechanism of muscarinic responses in *Xenopus* oocytes includes PIP_2 hydrolysis and the generation of IP_3 and its various metabolites [5,6,9,20]. We anticipated, therefore, that stimulation of cell membrane receptors will result in membrane electrical responses that are mediated in part by mobilization of calcium from cellular stores and in part by calcium influx through channels activated by IP_3 and/or its metabolites. This report demonstrates the validity of this hypothesis.

The calcium channels activated by acetylcholine do not appear to require membrane depolarization, neither are they inhibited by the classical antagonists of voltage-sensitive calcium channels (see also [4]). Moreover, Dascal et al. [21] have studied endogenous voltage-sensitive calcium channels in *Xenopus* oocytes and concluded that there is only a minimal population of such channels in this cell. We have found that although agonists do induce influx of labelled calcium into oocytes, depolarization of the cell membrane with 50 mM KCl does not (Shapira and Oron, unpublished). All these results strongly suggest that agonists or IP_3 do not activate voltage-sensitive calcium channels, at least not those inhibited by verapamil or diltiazem. In this respect, the neurotransmitter activates receptor-operated channels (ROCs). These may be 'true' ROCs, i.e. the receptor is an integral part of the channel (as in nicotinic receptors) or uses a guanine nucleotide-binding protein as an intermediary (as has been demonstrated by Birnbaumer and his collaborators [22] for ACh-activated potassium channels in the heart). On the other hand, these channels may be activated by IP_3 and/or its metabolites. In that case they should be redefined as 'second messenger-operated channels'. Although IP_3 and/or its metabolites appear to be the logical mediators for the opening of these calcium channels, on the basis of our experiments we cannot exclude at least a partial role of true ROCs.

The most striking finding of this report is that the response evoked by extracellular calcium is markedly potentiated when the cell calcium has been previously depleted by exposure to the agonist (either ACh or IP_3) in the absence of extracellular calcium. These results imply negative feedback of cellular calcium on calcium channels, chloride channels, or both. Preliminary experiments indicate that ACh-indicated ^{45}Ca uptake is stimulated by previous calcium depletion (Shapira and Oron, unpublished). This suggests that intracellular calcium regulates the activity of receptor-operated calcium channels in the plasma membrane. Similar characteristics were demonstrated for voltage-sensitive calcium channels [23–25]. Analogous inhibition of IP_3-activated calcium channels by high concentrations of calcium was also reported [19]. Gurney et al. [26], however, reported calcium stimulation of voltage-sensitive calcium channels in cardiac muscle. Hence, feedback inhibition of cellular calcium on its own entry may be a common, though not general property. Our data demonstrate that *Xenopus* oocytes may be a convenient system to investigate the molecular events involved in the activation of receptor-operated calcium channels.

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