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Changes in intracellular calcium and in membrane currents evoked by injection of inositol trisphosphate into *Xenopus* oocytes

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Intracellular calcium was monitored by the use of aequorin in voltage-clamped oocytes of *Xenopus laevis*. Injection of inositol trisphosphate (IP_3) into oocytes elicited slowly rising and decaying aequorin/calcium signals and produced oscillatory chloride membrane currents. These responses did not depend upon extracellular calcium, since they could be elicited in calcium-free solution and after addition of cobalt or lanthanum to block calcium channels in the surface membrane. We conclude that IP_3 causes the release of calcium from intracellular stores in the oocyte. Injections of calcium gave aequorin and membrane current responses that were more transient than those seen with IP_3.

Introduction

Oocytes of *Xenopus laevis* possess muscarinic receptors, which, in response to acetylcholine, give rise to slow oscillatory chloride currents (Kusano *et al.* 1977, 1982). Similar effects are also seen in response to serotonin and some other neurotransmitters, due to the expression of exogenous receptors in the oocyte following injection of messenger RNA from brain (Gundersen *et al.* 1982, 1984; Sumikawa *et al.* 1984). All of these oscillatory currents are dependent upon intracellular calcium ions (Parker *et al.* 1985*a*,*b*; Dascal *et al.* 1985), and can be mimicked by intracellular injection of inositol 1,4,5-trisphosphate (IP_3) (Oron *et al.* 1985; Miledi *et al.* 1986). Together with the fact that the oscillatory responses are seen in isotonic magnesium solution (Kusano *et al.* 1982) and that injection of calcium into oocytes elicits a chloride current (Miledi & Parker 1984; Dascal *et al.* 1985), these observations suggest that the oscillatory responses to neurotransmitters are activated through an internal messenger system involving IP_3 and calcium, similar to that proposed for various other cell types (Berridge & Irvine 1984; Hirasawa & Nishizuka 1985). In this scheme, receptor activation results in the increased hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce IP_3, which then causes mobilization of calcium from internal stores, and thereby activates the membrane chloride channels. We have examined this hypothesis by using the photoprotein aequorin to monitor changes in intracellular levels of free calcium (for review, see Blinks *et al.* 1981) together with recordings of the membrane currents induced by injection of IP_3, or calcium chloride, into voltage-clamped oocytes.

Methods

Experiments were made on native oocytes of *Xenopus laevis*, with electrophysiological techniques as described previously (Kusano *et al.* 1982; Miledi 1982). The oocytes were continuously perfused with Ringer solution at room temperature (*ca.* 24 °C). In addition to two microelectrodes for voltage clamping, each oocyte was penetrated by two other micropipettes to allow intracellular injection of aequorin, and IP_3 or calcium, by means of pneumatic pressure pulses (Miledi & Parker 1984). The IP_3 and aequorin pipettes were inserted about 200 μm apart, with the IP_3 pipette positioned either in the vegetal (white) hemisphere of the oocyte, or close to the equator separating the hemispheres. Aequorin was injected at a concentration of about 5 mg ml^{-1}, in a solution containing 750 mM KCl, 10 μM EDTA and 20 mM HEPES at neutral pH. IP_3 (Sigma Chemical Co.; D-*myo* inositol trisphosphate) was injected as a 1 mM solution, together with 50 μM EDTA and 5 mM HEPES at pH 7.0. Calcium injections were made from pipettes filled with 50 mM $CaCl_2$.

Light emitted from aequorin-loaded oocytes was collected by a ×10 long-working-distance objective lens (Leitz; numerical aperture 0.22) and projected onto a photomultiplier (EMI 9524B) operated at a potential of 800V. The anode current was measured via a current-to-voltage converter and filtered by a simple RC circuit with a time constant of 0.5 s.

Results

Oocytes were loaded with aequorin until close to the maximum volume which they could tolerate without damage (injection of *ca.* 50 nl). Soon after injection, most oocytes showed a resting light emission which increased when the membrane was hyperpolarized, or when the calcium concentration in the bathing solution was raised. This probably arose because of calcium influx around the sites of electrode impalements, or because of increased membrane permeability due to stretch (Miledi & Parker 1984). Therefore, oocytes were allowed to settle for about 30 min after loading, during which time the resting light emission usually declined and the input resistance increased.

Membrane currents and light responses to IP_3

Injection of IP_3 into oocytes generated membrane currents showing two components, which were inward at clamp potentials negative to −40 mV (figures 1 and 3), and were carried largely by chloride ions (Oron *et al.* 1985). A small but rapid increase in current coincided with the pressure pulse; this was followed, after some tens of seconds, by a larger, slowly rising and decaying current, which usually showed superimposed oscillations. These membrane current responses were accompanied by an increase in light emission by aequorin. However, this signal had a very different, and simpler, time course: the light began to rise at the time of the injection, reached a peak 10–100 s later, and then declined monotonically over several minutes. Increasing the dose of IP_3 gave larger current and aequorin responses, which showed slower rising and falling phases (figure 1*a*, *b*). The peak

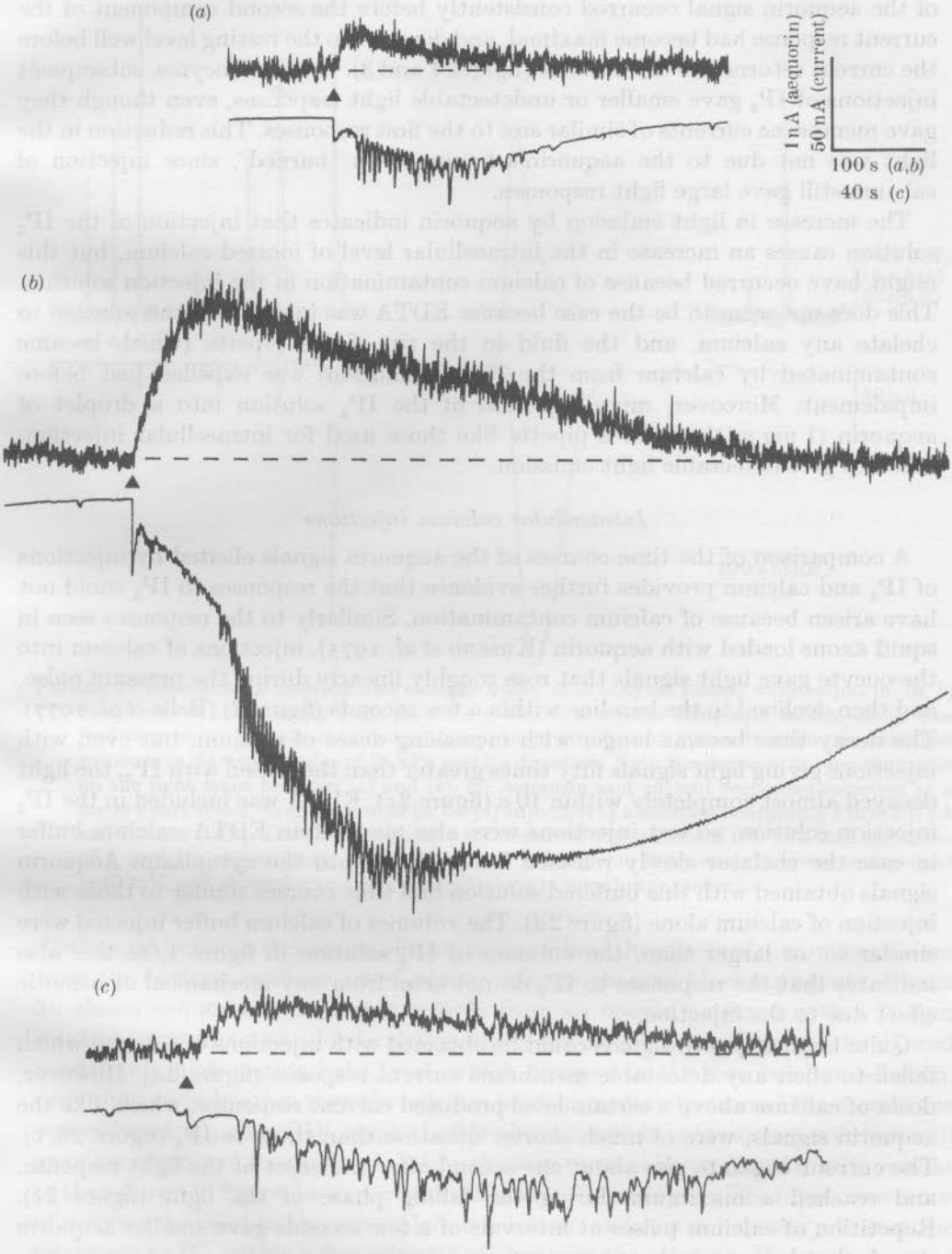

FIGURE 1. Aequorin light signals and membrane currents in response to injection of IP_3 into an aequorin-loaded oocyte. In this and other figures, the upper trace in each record shows light emission (shown as photomultiplier current) and the lower trace shows clamp current. Upward deflections correspond to increasing light and outward membrane current. The oocyte was clamped at a potential of −60 mV. Injections of IP_3 were made at times indicated by the arowheads, with pneumatic pressure pulses of about 100 kPa and 200 ms (*a*, *c*) and 1 s (*b*) duration, applied to a pipette containing 1 mM IP_3.

of the aequorin signal occurred consistently before the second component of the current response had become maximal, and decayed to the resting level well before the current returned to the baseline (figures 1 and 3). In a few oocytes, subsequent injections of IP_3 gave smaller or undetectable light responses, even though they gave membrane currents of similar size to the first responses. This reduction in the light was not due to the aequorin's having been 'burned', since injection of calcium still gave large light responses.

The increase in light emission by aequorin indicates that injection of the IP_3 solution causes an increase in the intracellular level of ionized calcium, but this might have occurred because of calcium contamination in the injection solution. This does not seem to be the case because EDTA was included in the solution to chelate any calcium, and the fluid in the tip of the pipette (which became contaminated by calcium from the Ringer solution) was expelled just before impalement. Moreover, micro-injection of the IP_3 solution into a droplet of aequorin (1 mg ml^{-1}), from a pipette like those used for intracellular injection, failed to give detectable light emission.

Intracellular calcium injections

A comparison of the time courses of the aequorin signals elicited by injections of IP_3 and calcium provides further evidence that the responses to IP_3 could not have arisen because of calcium contamination. Similarly to the responses seen in squid axons loaded with aequorin (Kusano *et al.* 1975), injections of calcium into the oocyte gave light signals that rose roughly linearly during the pressure pulse, and then declined to the baseline within a few seconds (figure 2) (Belle *et al.* 1977). The decay time became longer with increasing doses of calcium, but even with injections giving light signals fifty times greater than those seen with IP_3, the light decayed almost completely within 10 s (figure 2*c*). EDTA was included in the IP_3 injection solution, so test injections were also made of an EDTA–calcium buffer in case the chelator slowly released free calcium into the cytoplasm. Aequorin signals obtained with this buffered solution had time courses similar to those with injection of calcium alone (figure 2*d*). The volumes of calcium buffer injected were similar to, or larger than, the volumes of IP_3 solution in figure 1, so this also indicates that the responses to IP_3 do not arise from any mechanical or osmotic effect due to the injection.

Quite large aequorin signals could be obtained with injections of calcium which failed to elicit any detectable membrane current response (figure 2*a*). However, doses of calcium above a certain level produced current responses, which, like the aequorin signals, were of much shorter duration than those to IP_3 (figure 2*b*, *c*). The current began to rise about one second after the onset of the light response, and reached a maximum during the falling phase of the light (figure 2*c*). Repetition of calcium pulses at intervals of a few seconds gave smaller aequorin signals, but larger membrane currents.

Responses to IP_3 do not depend upon extracellular calcium

The light signals to injection of IP_3 could be due to release of calcium from intracellular stores, or to an influx of calcium from the extracellular medium. To

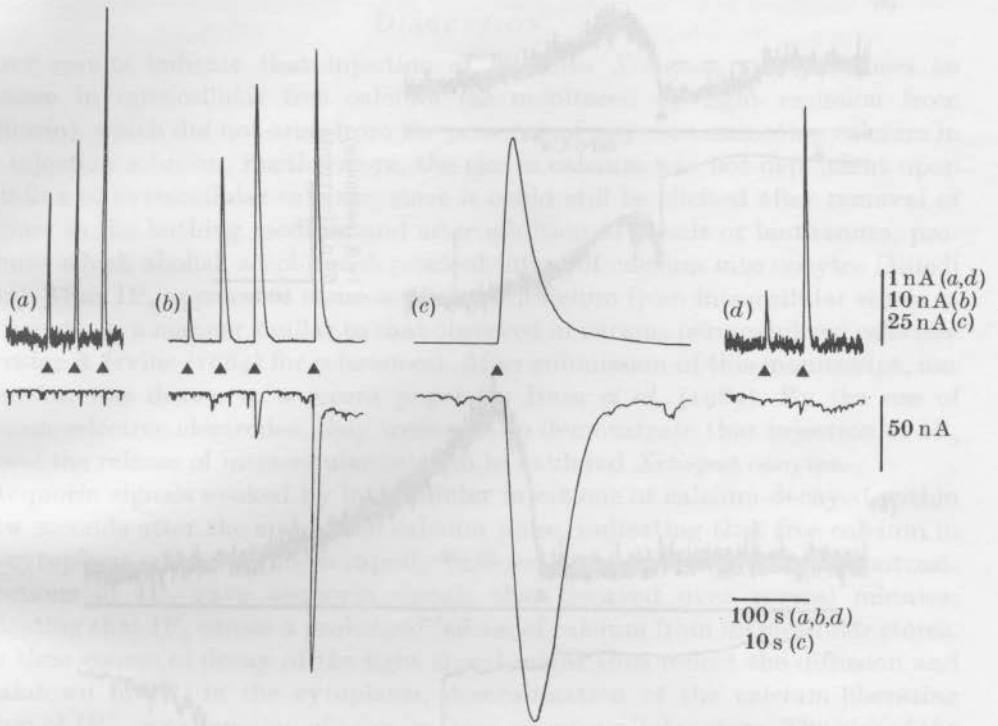

FIGURE 2. Injections of calcium and calcium buffer into oocytes loaded with aequorin. (*a*, *b*) Responses to increasingly large pressure pulses to a pipette filled with 50 mM $CaCl_2$. The first response in (*a*) was obtained with a pulse of 10 kPa and 50 ms duration, and the last response in (*b*) with a pulse of 25 kPa and 2 s duration. Note the decrease in recording gain on the light trace between (*a*) and (*b*). (*c*) Aequorin and current responses recorded at a faster chart speed. Same oocyte as (*a*, *b*). (*d*) Injection of a solution containing 1 mM EDTA and 0.5 mM $CaCl_2$. The two responses were obtained with pulses of 200 kPa and durations of 1 and 2 s respectively. Different oocyte from (*a*–*c*). Both oocytes were clamped at −40 mV, and showed spontaneous oscillations in membrane current.

distinguish between these possibilities, we examined the effects of removing calcium from the bathing medium, or of adding calcium-channel blocking agents. Figure 3*a* shows aequorin and current records from an oocyte perfused with a Ringer solution containing no added calcium and 2 mM magnesium. This oocyte showed a relatively high resting light level, which declined in the zero calcium solution, providing an indication that the calcium level immediately around the membrane was reduced. A pulse of IP_3 applied after about 2 min in zero calcium solution still gave aequorin and current signals like those in normal Ringer, and results similar to that shown in figure 3*a* were obtained in two other oocytes. The input resistance of oocytes declined in the zero calcium solution, and it was necessary to test the responses to IP_3 within a few minutes of changing the solution. However, oocytes frequently remained stable after addition of lanthanum (0.5 mM) or cobalt (10 mM) to the bathing medium, and these agents also failed to suppress the light and membrane current responses to IP_3 (see, for example, figure 3*b*), even though they block calcium channels in the oocyte (Miledi 1982; Parker *et al.* 1985*b*). Two

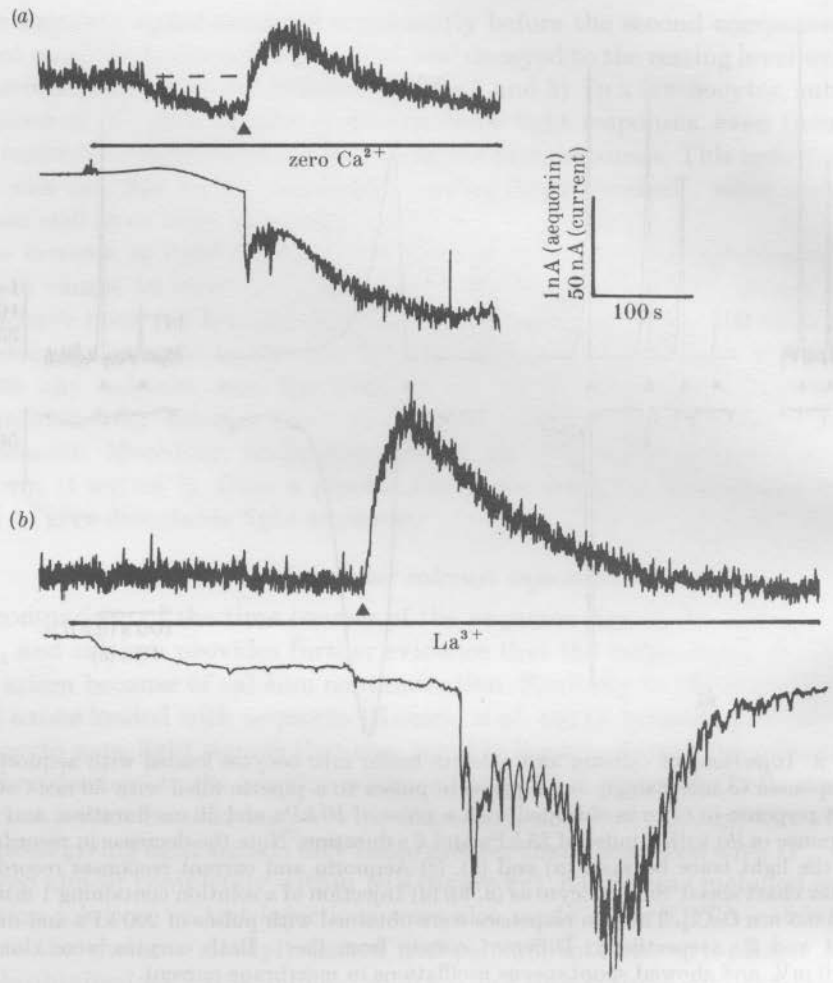

FIGURE 3. Effects of changes in extracellular ions on the aequorin signal and membrane current. In each record the oocyte was perfused with normal frog Ringer solution at the beginning of the trace, and the solution was then changed as indicated by the bars. Pulses of IP_3 (200 kPa, 100–200 ms) were applied as indicated by the arrowheads. (*a*) Perfusion with Ringer solution containing no added calcium and, additionally, 2 mM magnesium. (*b*) Normal Ringer solution including 0.5 mM lanthanum. Records from two oocytes, clamped at potentials of −40 mV.

oocytes examined with lanthanum, and three with cobalt, all showed responses, even when the blocking agents were perfused for up to twenty minutes before injecting IP_3.

As seen previously by R. Woodward and R. Miledi (unpublished observations), in some oocytes the addition of cobalt or manganese to the bathing medium elicited, by itself, oscillatory membrane currents resembling those that occur in response to IP_3 injection or to muscarinic activation (Kusano *et al.* 1982). A small transient increase in aequorin light was sometimes detectable during these responses, which had a time course similar to that produced by IP_3 injection.

Discussion

Our results indicate that injection of IP_3 into *Xenopus* oocytes causes an increase in intracellular free calcium (as monitored by light emission from aequorin), which did not arise from the presence of any contaminating calcium in the injection solution. Furthermore, the rise in calcium was not dependent upon an influx of extracellular calcium, since it could still be elicited after removal of calcium in the bathing medium and after addition of cobalt or lanthanum, procedures which abolish a voltage-dependent influx of calcium into oocytes (Miledi 1982). Thus IP_3 appears to cause a release of calcium from intracellular stores in the oocyte, in a manner similar to that observed in various permeabilized cells (see Berridge & Irvine (1984) for references). After submission of this manuscript, our attention was drawn to a recent paper by Busa *et al.* (1985). By the use of calcium-selective electrodes, they were able to demonstrate that injection of IP_3 caused the release of intracellular calcium in ovulated *Xenopus* oocytes.

Aequorin signals evoked by intracellular injections of calcium decayed within a few seconds after the end of the calcium pulse, indicating that free calcium in the cytoplasm of the oocyte is rapidly buffered and/or sequestered. In contrast, injections of IP_3 gave aequorin signals that decayed over several minutes, indicating that IP_3 causes a prolonged release of calcium from intracellular stores. The time course of decay of the light signal might thus reflect the diffusion and breakdown of IP_3 in the cytoplasm, desensitization of the calcium-liberating action of IP_3, or exhaustion of calcium from an intracellular store. The rise of the aequorin signal was also much slower following injection of IP_3 than with injection of calcium. It is not yet clear whether this occurs simply because IP_3, by diffusing away from the injection site, activates calcium release from a gradually increasing volume of cytoplasm, or whether it is caused by a slow onset of calcium liberation at each point.

As well as elevating the intracellular level of free calcium, injections of IP_3 activated oscillatory membrane currents. Several lines of evidence, including the abolition of oscillatory chloride currents by intracellular injection of a calcium chelator (Dascal *et al.* 1985; Parker *et al.* 1985) and the generation of a chloride current by calcium injection (Miledi & Parker 1984; Dascal *et al.* 1985), suggest that the currents produced by IP_3 arise because the resulting rise in intracellular calcium activates calcium-dependent chloride channels in the oocyte membrane. However, the time course of the calcium signal might then be expected to correspond to that of the membrane current, and this was not observed. In all oocytes examined, the aequorin signal reached a peak before the current had grown to a maximum, and sometimes the light emission was maximal before the second, oscillatory, phase of the current had begun to rise (see, for example, figures 1*a* and 3*b*). After the peak, the light signal decayed more rapidly than the current, and returned to the baseline while an appreciable current still remained. Also, we were unable to detect fluctuations in light emission corresponding to the marked oscillations in membrane current (see, for example, figure 1*c*).

The explanation for the lack of correspondence between the calcium signal and membrane current is, at present, unclear. One possibility involves the character-

istics of aequorin as a calcium monitor. The light signals from the oocyte probably arose largely from a region immediately around the tip of the IP_3 pipette, which would have been at a depth of several tens of micrometres from the membrane. In comparison to this, smaller elevations in the concentration of free calcium over a wide area close to the inner surface of the membrane may have contributed little to the signal, and could have escaped our detection. The power law relation that is observed under some conditions between the concentration of free calcium and light emission by aequorin (Blinks *et al.* 1981) might further accentuate this effect. Another possibility is that the membrane channels responsible for the chloride current may be modulated or activated by a substance other than calcium. For example, the channels might respond directly to IP_3, or to some intermediate in phosphoinositide metabolism.

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