Acetylcholine- and inositol 1,4,5-trisphosphate-induced calcium mobilization in *Xenopus laevis* oocytes

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Acetylcholine induces a complex electrical membrane response in *Xenopus laevis* oocytes. This response is mimicked, and probably mediated by injected inositol 1,4,5-trisphosphate. Oocytes prelabelled with ^{45}Ca released calcium in two phases, the second, slow phase exhibiting first order kinetics of release. Brief exposure of prelabelled oocytes to acetylcholine resulted in a significant increase in the rate of calcium release that returned to control values 2–3 min following the removal of the neurotransmitter. Intracellular injection of inositol 1,4,5-trisphosphate resulted in increased rate of calcium release similar to, but longer than that caused by acetylcholine. Experiments conducted on single oocytes permitted the investigation of the relationship between acetylcholine-induced and inositol 1,4,5-trisphosphate-induced calcium mobilization and the resulting electrical membrane response. Our data reinforce our previous suggestion that inositol 1,4,5-trisphosphate is the intracellular second messenger of the muscarinic membrane electrical response in *Xenopus* oocytes.

Inositol trisphosphate *Ca^{2+} mobilization* *Muscarinic response* (Xenopus) *Oocyte*

1. INTRODUCTION

A wide variety of stimuli that exert their effects by calcium mobilization will also enhance phosphoinositide breakdown. Berridge and his collaborators [1,2] have recently shown that the first biochemical event of receptor activation is the increased breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) to yield inositol 1,4,5-trisphosphate (IP_3). IP_3 has been shown to release calcium from intracellular non-mitochondrial pool(s) in both saponin-permeabilized cells [3–5] and isolated microsomes [6–8]. There is a large body of evidence that indicates that calcium-mobilizing stimuli increase IP_3 production and that IP_3 releases calcium from intracellular stores (review [9]). Several laboratories have demonstrated that intracellular injection of IP_3 mimics physiological responses to the corresponding stimuli [10–14]. However, all experiments showing IP_3-mediated calcium mobilization were conducted in either permeabilized cells or in cell-free systems from which the physiological effect of the stimulus was lost. We have chosen to study calcium mobilization in *Xenopus laevis* oocytes in which the response to IP_3 injections can be measured in an intact, acetylcholine (ACh)-responsive cell.

Brief exposure of *Xenopus* oocytes to ACh results in a complex membrane electrical response, consisting mainly of a prolonged depolarizing current [15,16]. It was shown that the depolarizing currents most probably result from calcium mobilization from intracellular stores and that ACh will increase calcium efflux from *Xenopus* oocytes [17]. We have shown that ACh induces an increase in oocyte IP_3 and that the injection of IP_3 mimics the ACh-induced D1, D2 and F responses in the *Xenopus* oocyte [10].

Here we present evidence that IP_3 induces calcium release from intracellular pools and that it is therefore probably the second messenger of the muscarinic response in *Xenopus* oocytes.

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2. MATERIALS AND METHODS

2.1. *Experimental animals*

Adult female *X. laevis* frogs were obtained from South African Snake Farm and maintained on beef liver diet at 18–20°C in a 14/10 h light-dark cycle.

2.2. *Oocyte preparation and ^{45}Ca labelling*

Pieces of ovary were excised and stage 5 and 6 oocytes were obtained under sterile conditions as described [14]. The cells were rinsed and incubated overnight at 20°C in a sterile ND 96 cell culture medium containing 0.1 mCi/ml ^{45}Ca. The concentration of calcium in the medium was 0.5 mM, yielding a final specific activity of calcium of 200 μCi/μmol.

2.3. *^{45}Ca efflux measurements*

The efflux of radiolabelled calcium was measured in the following way: a single oocyte was placed in a 0.4 ml Lucite perfusion bath and continuously perfused with the help of a peristaltic pump at a rate of 1 ml/min. ACh was added to the perfusate for the desired period of time in ACh-induced efflux experiments, or IP_3 was injected in IP_3-induced efflux experiments (see below). The perfusate was collected with a fraction collector every 20–30 s. Samples of perfusate were counted in 4.5 ml Hydroluma scintillator in a Packard scintillation counter. At the end of each experiment oocytes were homogenized in 0.4 ml of medium and the remaining radioactivity was determined.

2.4. *Intracellular injections*

Small diameter thin wall capillaries were pulled to a fine tip with a conventional vertical Narashige puller set at 14 mA. The tip was manually broken to a 5–10 μm diameter and the micropipette filled with light silicone oil. The needle of a 0.5 μl Hamilton syringe was inserted into the micropipette and the tip was filled with 0.5–1.0 mM solution of IP_3 by aspiration. The syringe was attached to a Narashige micromanipulator and the micropipette inserted into an oocyte placed in the perfusion bath. The contents of the pipette were injected by manual pressure on the syringe piston. We could inject volumes as small as 2.5 nl, verified occasionally by injection into an oil droplet under a microscope. In a routine experiment 5–10 nl were injected into a single oocyte.

2.5. *Electrophysiology*

The electrophysiological set-up was detailed in [14]. The cell membrane potential was clamped at a desired value and membrane current was followed.

2.6. *Solutions*

ND 96 enriched medium composition was: 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM Hepes, 1 mM sodium pyruvate, 1 mM NaH_2PO_4, 100 U/ml penicillin, pH 7.4. The composition of OR-2 solution was reported [14].

2.7. *Chemicals*

ACh, Hepes, sodium pyruvate and penicillin were products of Sigma, Israel. IP_3 was either isolated from fresh rabbit or human erythrocytes as described [19] or purchased from Amersham. $^{45}CaCl_2$ was purchased from Amersham and Hydroluma from Lumac. All other chemicals were of analytical grade.

3. RESULTS

3.1. *ACh-induced calcium efflux*

Labelled oocytes appeared to release calcium from two pools – a rapidly exchangeable pool and a slowly exchangeable pool that released calcium in apparent first order kinetics. We challenged oocytes with ACh following 10–15 min of wash, after the first rapid efflux phase was essentially over. The effect of ACh could not be detected when the first phase of efflux was still predominant. ACh (10 μM), applied for 2 min, significantly increased the rate of ^{45}Ca efflux (fig.1a). The onset of the increased efflux was rapid and occurred within seconds of the exposure to ACh. When calculated as a percentage of the total intracellular calcium remaining after the removal of the rapidly released pool, the unstimulated rate of calcium efflux was 0.8 ± 0.2%/min (mean ± SE, $n = 11$) and the stimulated mean rate of release during ACh stimulation 1.7 ± 0.2%/min ($n = 9$). The duration of the stimulated release was longer than the duration of ACh exposure (5.4 ± 0.2 min). These results are summarized in table 1. The ratio of stimulated to unstimulated efflux at the peak of the response was much higher than the calculated mean and ranged routinely between 3–5-fold. Occasionally much higher values were measured.

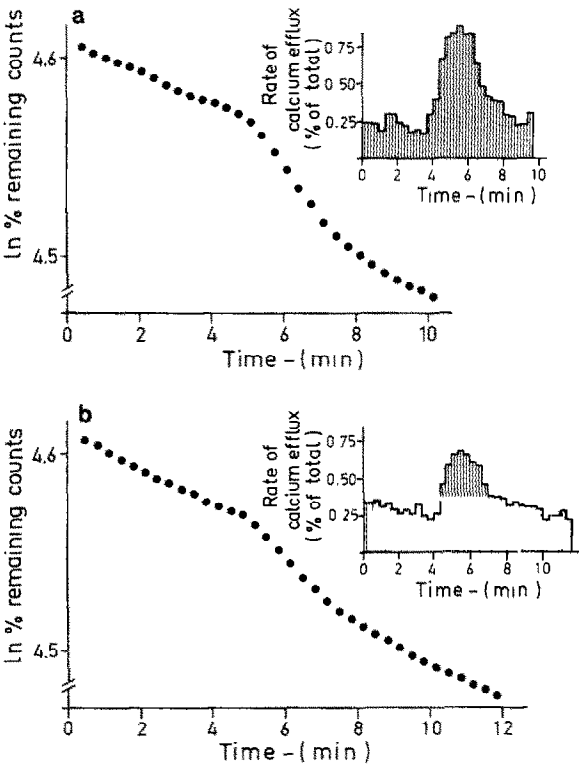

Fig.1. ACh and IP_3 effect on ^{45}Ca efflux in a single oocyte. (a) A single oocyte was preloaded with ^{45}Ca (see section 2) and exposed to 10 μM ACh for 2 min (between min 4 and 6) following 15 min of perfusion ($t=0$). The efflux of ^{45}Ca was monitored as described in section 2. (b) A single oocyte from the same batch was injected at $t=4$ min with 5 pmol IP_3 following 15 min of perfusion ($t=0$). ^{45}Ca efflux was monitored as in (a). Data are presented as a semi-logarithmic plot of Δ remaining counts vs time. Oocytes were exposed to ACh or IP_3 during a linear first order phase of ^{45}Ca efflux. Plots of rate of efflux (as a Δ of total counts at each time point) vs time are presented in the insets.

3.2. *IP_3 induced calcium release*

Intracellular injection of IP_3induced release of calcium similar to that caused by ACh. The onset of increased release, as well as the onset of the ensuing chloride current, were rapid (i.e in less than 20 s). In general, the IP_3-induced increase of efflux was more gradual (mean increase in efflux 1.2 ± 0.3 ($n=7$) vs 1.7 ± 0.2%/min, see table 1). The IP_3-induced release lasted, however, longer than that of ACh (7.4 ± 0.9 vs 5.4 ± 0.6 min, see table

Table 1

ACh- and IP_3-stimulated calcium efflux from *Xenopus* oocytes

	Control	ACh	Control	IP_3
Mean rate of ^{45}Ca efflux (% of total/min)	0.8±0.2	1.7±0.2	0.8±0.2	1.2±0.3
Mean increase in efflux (% over control)		112		50
Peak increase in efflux (% over control)		285		119
Duration of increased efflux (min)		5.4±0.6		7.4±0.9
Total stimulated efflux (% of total)		4.6		3.1

Oocytes were labelled with ^{45}Ca as described in section 2. Single oocytes were assayed for unstimulated calcium efflux (controls) and challenged with either 10 μM ACh for 2 min or injected with 5 pmol IP_3. The basal rate of efflux was between 200 and 600 cpm/min and the content of ^{45}Ca of a single oocyte varied between 6000 and 10000 cpm. The mean rate of efflux was calculated by dividing the total efflux during the response by the overall duration of the response. Results are presented as mean ± SE of 7–11 experiments in oocytes of 5–8 different frogs

1), resulting in a slightly smaller total release (3.1 vs 4.6% of total oocyte calcium). A representative experiment is shown in fig.1b. In some frogs, both the ACh-induced chloride current and ^{45}Ca efflux were several times larger than in oocytes from most of the animals. An example of such a large response to both ACh and IP_3 is presented in fig.2.

3.3. *The correlation between calcium release and the evoked chloride current*

Our experimental set-up allowed simultaneous monitoring of the ACh- or IP_3-induced calcium release and the corresponding membrane electrical response under voltage clamp conditions. In general, the increase in chloride current lasted longer than the exposure to ACh and followed the increase in calcium efflux. In many experiments, however, increased calcium efflux was still significant after the chloride current returned to control values (see fig.2). The similarity of the ACh- and IP_3-induced membrane electrical response and of the concurrent calcium release could be documented by monitoring both responses in the same oocyte (see fig.2a,b). Applying supramaximal stimuli of both ACh and IP_3, virtually identical membrane electrical responses and calcium release profiles were obtained.

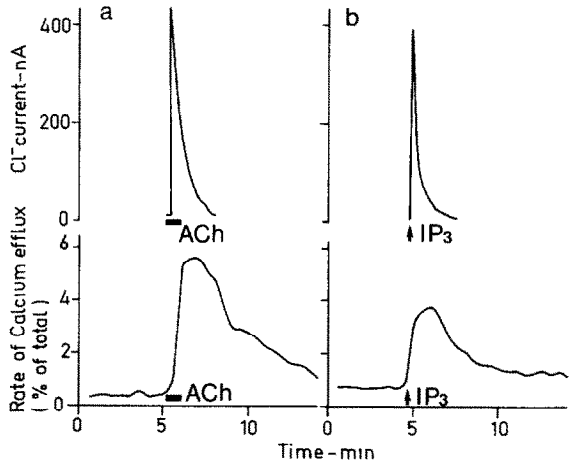

Fig.2. ACh and IP_3 effect on chloride current and ^{45}Ca efflux in a single oocyte. (a) A single oocyte was preloaded with ^{45}Ca (see section 2) and exposed to 10 μM ACh for 2 min following 15 min of perfusion. The membrane electrical response (upper panel) and the efflux of ^{45}Ca (lower panel) were monitored simultaneously. (b) A single oocyte from the same batch was injected with 5 pmol IP_3 following 15 min of perfusion. Chloride current and ^{45}Ca efflux were monitored as in (a).

4. DISCUSSION

ACh induced a significant increase in calcium efflux from ^{45}Ca-labelled oocytes. Dascal et al. [17] have shown preliminary evidence of ACh-induced increase of calcium efflux in *Xenopus* oocytes. They have also demonstrated that calcium depletion and repletion correlated well with the magnitude of the corresponding muscarinic chloride current and that intracellular calcium injections induce chloride currents similar to those induced by ACh [17]. In conclusion, the activation of muscarinic receptors in the oocyte results in the mobilization of intracellular calcium which, in turn, activates chloride channels in the cell membrane.

ACh released approx. 5% of its slowly exchanging calcium when challenged for 2 min with ACh. Dascal et al. [17] have shown that under calcium-free conditions the membrane electrical responses to successive applications of ACh diminished rapidly, reaching 10% of the control response by the third application. Rough calculation will indicate that the store of calcium available for ACh-induced release would not exceed 10–20% of total oocyte calcium remaining after the rapidly exchangeable pool was depleted. In medium containing calcium, however, this pool is continuously repleted, thus allowing repeated responses to ACh. In oocytes of the few frogs that exhibited a much larger response (see fig.2) the available calcium pool must be much more extensive. It would be interesting to examine the intracellular morphology of these oocytes.

The injection of IP_3 into the oocyte resulted in an increase in calcium efflux comparable and similar to that caused by challenge with ACh. We assumed that we could compare ACh- and IP_3-induced releases of ^{45}Ca, since both agents were used at their saturating concentrations. The small differences in the kinetics of ^{45}Ca release by the two agents might be related to differences in the site of IP_3 generation by ACh, as compared to the site of intracellular injection of IP_3. The kinetics of the onset of the ACh- or IP_3-induced calcium efflux were rapid, within 15–30 s of the exposure. This was similar to the delay in the onset of the in-

creased chloride current and was compatible with the assumption that calcium mobilization precedes and induces chloride gating. In most experiments, increased calcium efflux was observed after ACh was removed and the chloride current returned to control rates. This may signify that the decay of the chloride current may be regulated by a different mechanism, while the intracellular calcium concentration is still significantly elevated (see fig.2).

Other laboratories have demonstrated that IP_3 releases non-mitochondrial calcium from saponin-permeabilized cells [3–5] and from isolated, calcium loaded microsomes [6–8]. Recently, Busa et al. [18], using an intracellular calcium-sensitive electrode, have reported that iontophoretic injection of IP_3 induced intracellular calcium release in mature *Xenopus* oocytes. They have related their findings to the wave of calcium release and depolarization that accompanies oocyte fertilization. There is no proof, however, that the IP_3-sensitive calcium pool described by Busa and his collaborators is identical to the ACh-mobilized pool. We have shown previously, however, that the mature *Xenopus* oocyte is no longer responsive to neurotransmitter stimulation [19] and cannot serve as a model for the elucidation of the molecular mechanism of the activation of neurotransmitter receptors.

Our previous work has shown that ACh induces an increase in oocyte IP_3 [10], that IP_3 mimics the ACh response in the oocyte [10] and that ACh induces calcium efflux in the oocyte. Here we demonstrate IP_3-mediated calcium efflux in an intact, ACh-sensitive cell. Further experiments are required to investigate the identity of calcium pools in the *Xenopus* oocyte that participate in the neurotransmitter-induced and in fertilization-induced responses.

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