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Free intracellular cations in echinoderm oocytes and eggs

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Abstract. The concentrations of Ca^{2+}, Na^+ and H^+ in echinoderm oocytes and eggs were measured during maturation and activation using ion-selective microelectrodes. In both oocytes and eggs, from three species of starfish and two species of sea urchin, the resting level of cytosolic Ca^{2+} was about 10^{-7} *M*. We did not detect any change in Ca^{2+} concentration either during hormone-induced oocyte maturation (starfish) or during egg activation (starfish and sea urchin) induced by spermatozoa or chemical agents. During 1-methyl-adenine induced maturation of starfish oocytes the intracellular level of Na^+ increased from 12–35 m*M* to 40–90 m*M*, while the pH changed from 6.6–6.8 to 7.0–7.2. Aged oocytes, with intact germinal vesicles, also had elevated levels of Na^+ and pH.

Key words: Echinoderm oocytes, eggs, maturation, activation, cations, ion selective microelectrodes

Introduction

In echinoderm oocytes and eggs, levels of free intracellular Ca^{2+} have been estimated using the photoprotein aequorin (Moreau et al. 1978; Steinhardt et al. 1977; Eisen and Reynolds 1984; Eisen et al. 1984) and a fluorescent dye, Fura-2 (Poenie et al. 1985), whereas in *Xenopus* oocytes $[Ca^{2+}]_{in}$ was measured directly using ion-selective micro-electrodes (Busa and Nuccitelli 1985). Intracellular levels of Na^+ and H^+ have been calculated by a variety of biochemical and electrical techniques (Shen 1983; Meijer and Guerrier 1984; Shen and Burgart 1985; Payan et al. 1981) and Johnson et al. (1976) postulated that a Na^+/H^+ exchange mechanism was involved in the cytoplasmic alkalinization of sea urchin eggs at fertilization.

The biochemical and luminescent indicator techniques are particularly insensitive for measuring resting cation levels and in discriminating regional versus total changes in ion activities. Although it is generally accepted that there is a transient increase in $[Ca^{2+}]_{in}$ at oocyte de-repression, there is disagreement over its magnitude (Moreau et al. 1978; Eisen and Reynolds 1984) and whether it is restricted to the cortical layer (Busa and Nuccitelli 1985). Furthermore, although a pH change has been reported to occur during the maturation of *Xenopus* oocytes (Lee and Steinhardt 1981; Cicirelli et al. 1983), Johnson and Epel (1982) and Peaucellier and Doree (1981) failed to detect such a change in the starfish oocyte.

The purpose of the present paper was to measure the concentrations of Ca^{2+}, Na^+ and H^+ in echinoderm oocytes and eggs during de-repression using ion-selective micro-electrodes. In addition to the sensitivity of this method for measuring cations, an intrinsic advantage is that changes in membrane potential are measured simultaneously, and these may be used as temporal markers for other cellular events associated with de-repression (Dale et al. 1978, 1979; Dale and Santella 1985). Gametes of the sea urchin and starfish were selected because they are morphologically similar and yet the strategy of de-repression in the two cases is different. In the former case the spermatozoon triggers egg activation, whereas in the latter, oocyte de-repression is initiated by the hormone 1-methyladenine (Kanatani 1975).

Materials and methods

Experiments were carried out using gametes of the sea urchins *Paracentrotus lividus* and *Sphaerechinus*

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granularis and of the starfish *Astropecten aurantiacus, Marthasterias glacialis* and *Ophidiaster ophidianus.* All animals were collected locally from the Bay of Naples. Gametes were obtained by dissection; oocytes and eggs were kept in natural sea water and the spermatozoa stored "dry". Experiments were carried out at ambient temperature (18–21 °C), for the most part in natural sea water, with the gametes on glass slides. In the majority of cases the cells were impaled with their jelly layers intact.

Ion-selective micro-electrodes were prepared from 2 mm o.d. capillary glass (Clark Electromedical, Reading, UK) with tips of 1 to 2 µm diameter. First the glass was pulled, on a horizontal electrode puller, to form a conventional micro-electrode. The tips were immersed into a freshly prepared solution of 2.5% (v/v) *tri-n*-butylchlorosilane (Pfaltz and Bauer, Conn.) in 1-Chloronaphthalene (Pfaltz and Bauer, Conn.) for about 25 s and then placed in an oven at 150–200 °C for 2 h. At the light microscope, a drop of the respective ligand was inserted into the back of the electrode; from where it was worked down into the tip, partly by capillary action and often helped using a cats whisker (Thomas 1978). To minimize the column length of the ligand and therefore to reduce the micro-electrode resistance, a second micro-pipette made from 1 mm glass (Clark Electromedical, Reading) and filled with 1.8 *M* K Citrate or 3 *M* KCl was inserted into the back of the ligand containing pipette (Ujec et al. 1979). The tip of this second electrode was pushed into the ligand to within 50–100 µm of the tip of the outer pipette and then fixed into position at the collar using epoxy resin.

Ion selective electrodes were calibrated with standard solutions before and after each experiment. The source of the ligand and the composition of the calibration solutions are as follows. Ca^{2+} ligand (Prof. Simon, Zürich); buffers of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M Ca^{2+} were prepared according to the formula of Gorman et al. (1984). Na^+ ligand (Prof. Simon, Zürich and Fluka Buchs, No. 71732); solutions of 10 m*M*, 30 m*M* and 50 m*M* NaCl containing 120 m*M* KCl and 10 m*M* Hepes (B.D.H., England) at pH 7.0. H^+ ligand (Prof. Simon, Zürich and Fluka Buchs, No. 91660); intracellular-like solutions, at pH values of 5.5 to 7.5 monitored continuously using a conventional pH meter. The calcium selective electrodes displayed little hysteresis on calibration and had Nernstian slopes above pCa 6. The voltage response between 10^{-8} M and 10^{-7} M Ca was usually 4–5 mV.

Cells were first penetrated by the ion-selective electrode by a light mechanical shock and then by a second conventional micro-electrode filled with 1.8 *M* K Citrate or 3 *M* KCl, to record membrane potential. To ensure both electrodes were indeed intracellular current pulses were routinely passed through the voltage recording electrode and deflections measured on the ion selective electrode. The signals were stored on FM tape and observed on a storage oscilloscope at which stage the membrane potential was subtracted from the ion selective electrode potential.

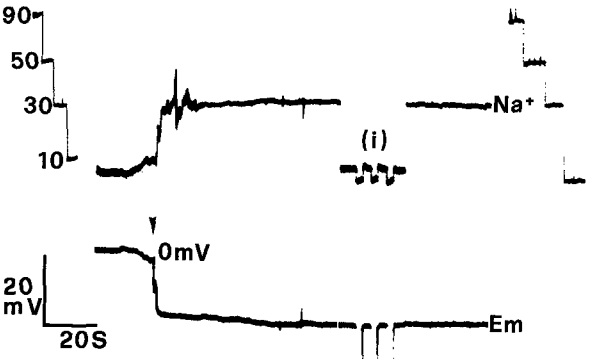

Fig. 1. The concentration of Na^+ in a germinal vesicle stage oocyte of the starfish *Astropecten aurantiacus.* The *upper trace* (Na^+) is the potential of the ion selective electrode while the *lower trace* (E_m) shows the cell's resting potential. First the ion selective electrode is placed in the oocyte by mechanical thrust and then, at the *arrow*, the conventional electrode penetrates the cell by oscillation using the negative capacitance of the pre-amplifier. The cell E_m is then subtracted from the *top trace* to give cell Na^+. The calibration points before and after the experiment are in standard solutions (m*M*). To check both electrodes are indeed intracellular current pulses were routinely passed through the voltage electrode and observed on the ion selective electrode (*i*)

Results

Starfish oocytes

We measured $[Na^+]_{in}$ in 16 oocytes and 2 mature eggs. Germinal vesicle stage oocytes from freshly opened animals had levels of $[Na^+]_{in}$ ranging from 12 to 35 m*M* ($n = 10$, Fig. 1), whereas mature eggs, aged oocytes (5–6 h in vitro) and oocytes from animals that had been previously opened had $[Na^+]_{in}$ levels of 40 to 90 m*M* ($n = 6$, see Fig. 2). Freshly prepared germinal vesicle stage oocytes had an intracellular pH ranging from 6.6 to 6.8 ($n = 23$, Fig. 3A) whereas mature eggs and aged oocytes had an intracellular pH in the range 7.0 to 7.2 ($n = 10$, Fig. 2B). In several experiments we were able to follow the maturation process and consequent increase in intracellular pH without the electrodes being dislodged (Fig. 4).

Measurements of intracellular Ca^{2+} levels were carried out on 15 starfish oocytes and eggs. Most

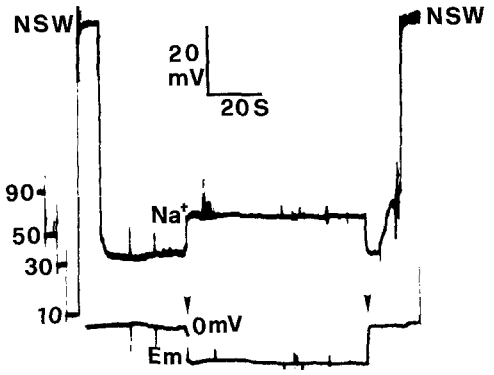

Fig. 2. Intracellular concentration of Na^+ in a mature egg of the starfish *Astropecten aurantiacus*. The *top trace* shows the voltage level of the ion selective electrode in the calibration solutions (m*M*) then in sea water (*NSW*) and finally in the cell. At the *arrow* the conventional voltage electrode is inserted in the cell to measure resting potential (E_m) and this voltage subtracted from the *top trace* to give cell Na^+. At the second *arrow* the potential recording electrode is withdrawn from the cell, causing an equivalent loss of voltage on the ion-selective trace, and then later this second electrode is withdrawn and its voltage recorded in sea water (*NSW*)

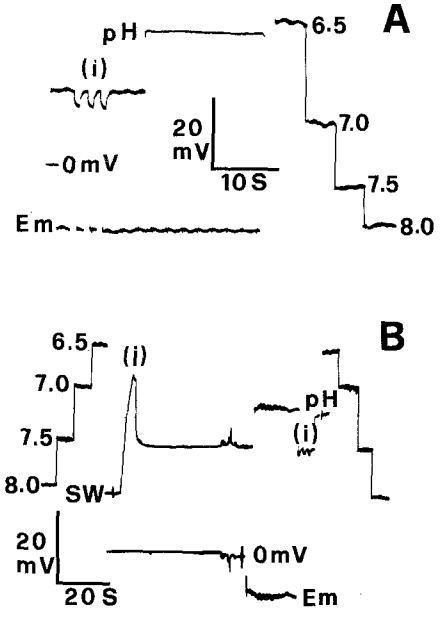

Fig. 3. **A** Intracellular pH in a germinal vesicle stage oocyte of the starfish *Ophidiaster ophidianus*. The *top trace* shows the voltage level of the pH electrode, while the *bottom trace* shows the cell resting potential (E_m). At the beginning of the experiment current pulses are passed through the voltage electrode and detected on the ion-selective electrode verifying that both electrodes are indeed intracellular (*i*). After subtracting the E_m, the pH of the cell is measured as 6.6. Calibration in standard pH solutions are shown after the experiment. **B** Intracellular pH in a mature egg of the starfish *Marthasterias glacialis*. The *top trace* shows the pH electrode first in sea water (*SW*), in the jelly layer of the egg (*J*) and finally intracellular. Subtracting the cell E_m (*lower trace*) gives the cellular intracellular pH (≈ 7.2). Calibration curves for the ion-selective electrode are shown before and after the experiment. Test pulses are also shown (*i*)

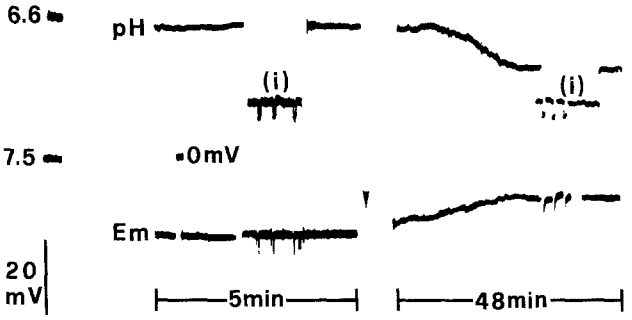

Fig. 4. Continuous recording of intracellular pH (*upper trace*) and membrane potential (*lower trace*) in an oocyte of *Astropecten aurantiacus* during 1-methyl adenine induced meiotic maturation. Calibration of the electrode is shown before the experiment. During the experiment, to check the intracellular location of both electrodes, current pulses passed through the voltage electrode are recorded on the ion selective electrode (*i*). Note the gradual membrane depolarization and increase in intracellular pH. 1-Methyladenine at 10^{-6} M was added at the *arrow*, and 30 min later the recording was re-initiated

cells had resting levels around 10^{-7} M. In five experiments we added the maturation hormone 1-methyladenine to impaled germinal vesicle stage oocytes and followed the $[Ca^{2+}]_{in}$ over a course of 1 h. During oocyte maturation, as evidenced by the breakdown of the germinal vesicle there was no significant variation in $[Ca^{2+}]_{in}$. In three experiments there was an apparent gradual depolarization of the ion-selective electrode, however, re-calibration following the experiment showed that it was, in fact, due to a polarization artifact. In six experiments using the mature starfish egg we induced the cortical reaction by insemination, by addition of 20 μM of the calcium ionophore A23187, or by temporarily replacing the sea water with a 1 *M* solution of urea. In all cases, we observed the characteristic activation potential, which is a slow bell-shaped depolarization and repolarization of 10 to 20 mV, however without any change in intracellular Ca^{2+}, which remained at values of about 10^{-7} M (Fig. 5).

Sea urchin eggs

There are numerous reports in the literature giving measurements of pH (see reviews, Shen 1983; Busa and Nuccitelli 1984) and $[Na^+]_{in}$ (Payan et al. 1981; Shen and Burgart 1985) in sea urchin eggs during de-repression. For this reason we concentrated on measuring changes in $[Ca^{2+}]_{in}$ in sea urchin eggs during insemination. In all we recorded $[Ca^{2+}]_{in}$ from 15 eggs. Resting levels were about 10^{-7} M and this also applied to germinal vesicle stage oocytes (Figs. 6 and 7). Insemination of oocytes or eggs resulted in depolarization of the membrane and the

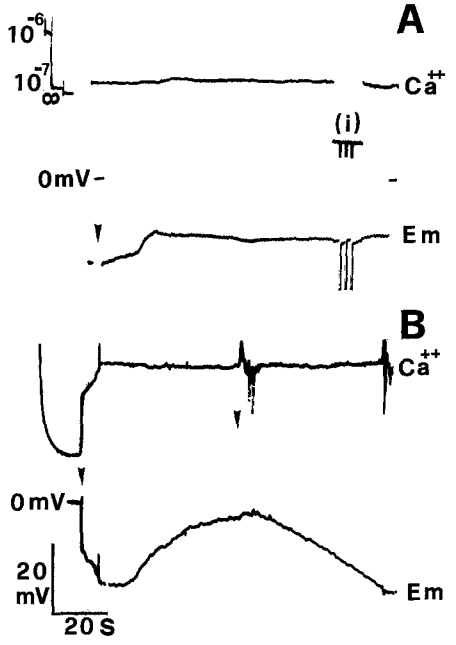

Fig. 5 A and B. Cytosolic Ca^{2+} in starfish eggs (*Marthasterias glacialis*) during the cortical reaction. **A** The *upper trace* shows the voltage level of the Ca^{2+} sensitive electrode, while the *lower trace* shows the cells resting potential (E_m). At the *arrow* the egg was inseminated and gave rise to the characteristic slow bell-shaped depolarization, called the fertilization potential, and a fertilization membrane. To check both electrodes are indeed intracellular current pulses are passed through the voltage electrode and recorded on the ion-selective electrode (*i*). Calibration is shown before the experiment. **B** Ca^{2+} and E_m in an egg induced to undergo the cortical reaction following exposure to 1 *M* Urea. The *upper trace is the* Ca^{2+} selective electrode, the *lower trace* E_m. At the *first arrow* the voltage electrode penetrates the cell and the voltage then subtracted from the Ca^{2+} electrode; this now gives the intracellular Ca^{2+} concentration which is 10^{-7} M. The cell is parthenogenetically activated by the Urea and gives rise to a bell-shaped depolarization. At the *second arrow* the faraday cage was opened to observe the egg which was in the process of elevating a fertilization-like membrane. Note that Ca^{2+} did not change

formation of multiple penetration cones (in the former case) or the elevation of a fertilization membrane (in the latter case), but without significant changes in the $[Ca^{2+}]_{in}$ (Fig. 6). Again in many experiments a slow drift in the ion-selective electrode appeared to indicate a slight increase in $[Ca^{2+}]_{in}$, however re-calibration of the electrode indicated that the drift was probably the result of polarization of some component in the circuit. To determine whether our electrodes were in fact able to measure changes in $[Ca^{2+}]_{in}$ in four experiments we added lethal doses of the ionophore A23187 (50 to 100 μM). An example is shown in Fig. 7. It can be seen that the membrane depolarized giving rise to a fertilization-like potential and this was followed by a 100 fold increase in the level of intracellular $[Ca^{2+}]_{in}$.

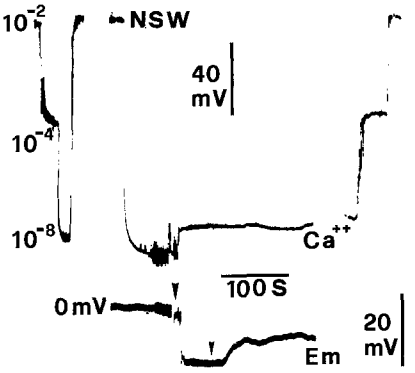

Fig. 6. Cytosolic Ca^{2+} in an oocyte of the sea urchin *Sphaerechinus granularis* during insemination. The upper trace shows the voltage level of the Ca^{2+} electrode, first in standard calibration solutions (*M*) then in sea water (*NSW*) and finally on penetrating the cell. At the *arrow* the conventional voltage recording electrode penetrates the cell and the E_m is subtracted from the ion-selective electrode. The resulting value, about 10^{-7} M, is cell Ca^{2+}. At the *second arrow* the oocyte was inseminated with 10^7 sperm/ml, the cell depolarized and several fertilization cones were observed. Following the experiment the Ca^{2+} electrode was re-calibrated. Note there was no increase in Ca^{2+}

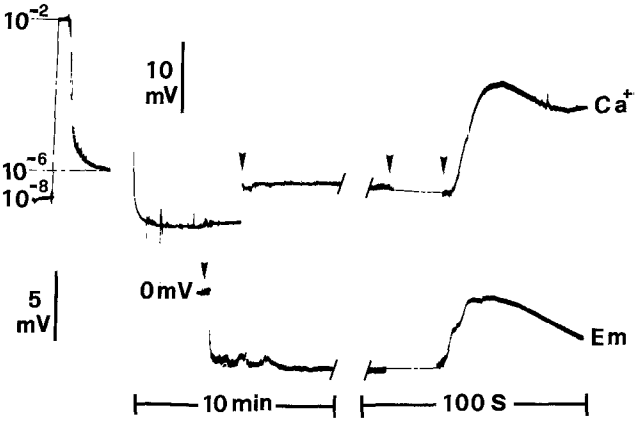

Fig. 7. Cytosolic Ca^{2+} in an egg of the sea urchin *Paracentrotus lividus*. The *upper trace* shows the Ca^{2+} selective electrode in the calibration solutions (*M*) and then in the cell. At the *first arrow* the conventional voltage electrode penetrates the cell and the recorded E_m is then later subtracted from the *top trace* (*second arrow*). The resting Ca^{2+} is about 10^{-7} M. Between the 3rd and 4th *arrows* the bath was perfused with sea water containing 100 μM of the divalent cation ionophore A23187. This lethal dose induced a depolarization of the cell E_m followed 5 s later by an increase in cytosolic Ca^{2+}

Discussion

A variety of studies have implicated Ca^{2+} as a signal in the regulation of cellular activity, however its mechanism of action remains unclear. As a first approach it is necessary to measure levels of intracellular Ca^{2+} and correlate any changes with cellular

behaviour. At present there are two techniques available. The first employs chemical indicators sensitive to Ca^{2+}; the second is the use of ion-selective intracellular micro-electrodes. In the present study we selected the second method which has the advantage of being a direct determination and in addition will identify localized changes in ions. A major drawback with the indicator method is that, by definition, the dyes chelate Ca^{2+} and therefore may themselves influence the progression of a presumptive ionic signal.

Previous authors using fluorescent or luminescent dyes have estimated the resting Ca^{2+} concentration in echinoderm eggs and oocytes to vary between 10^{-6} M and 10^{-7} M (Steinhardt et al. 1977; Moreau et al. 1978; Eisen and Reynolds 1984; Eisen et al. 1984; Poenie et al. 1985). By using ion-selective micro-electrodes we have shown that the resting $[Ca^{2+}]_{in}$ is, in fact, about 10^{-7} M. This is comparable to the resting Ca^{2+} level in *Oryzias* embryos (Schantz 1985) also measured by ion-selective electrodes and is almost two orders lower than the values reported for *Xenopus* embryos (Rink et al. 1980; Busa and Nuccitelli 1985). Within minutes of penetration the Ca^{2+} sensitive electrodes used in our experiments reached stable voltages representing 10^{-7} M levels. It is unlikely, therefore, that there was any significant leakage of Ca^{2+} into the cell around the tip of the electrode, despite its large diameter.

In the present report we did not detect any increase in $[Ca^{2+}]_{in}$ either at oocyte maturation, fertilization, or parthenogenetic activation. Our ion selective electrodes were probably placed at least 5 μm from the surface and were capable of detecting intracellular Ca^{2+} as shown by the addition of lethal amounts of the ionophore A23187 (see Fig. 7). We may consider two possibilities as to why we did not detect Ca^{2+} signals in eggs. First, the Ca^{2+} wave may indeed be localized to the cortex in which case an attempt must be made to record from within 1–2 μm of the plasma membrane. Alternatively, perhaps the time course of the Ca^{2+} transient is different to that indicated by the dye methods, being released from its source and bound by its receptor in fractions of a second. Thus, giving rise to a transient electrical potential shorter than the time constant of the system. In this case, ion selective micro-electrodes will not detect such a rapid change.

A second ionic signal implicated in the activation of sea urchin eggs is an increase in intracellular pH (Shen and Steinhardt 1978) which correlates temporally, and is most probably linked, to an increase in $[Na^+]_{in}$ (Payan et al. 1981, 1983; Shen and Burgart 1985). Several authors have reported that maturation of starfish oocytes is not accompanied by an increase in intracellular pH (Johnson and Epel 1982; Peaucellier and Doree 1981), whereas an increase in $[Na^+]_{in}$ and pH has been observed during the maturation of amphibian oocytes (Morrill 1965; Lee and Steinhardt 1981; Cicirelli et al. 1983; see also Schlichter 1983; Baud and Barish 1984). In contrast to the previous studies on starfish oocyte maturation, but in agreement with those on amphibian oocyte maturation, our results show that starfish oocyte maturation is accompanied by an increase in cytoplasmic pH and $[Na^+]_{in}$.

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