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KCl-Induced Calcium Rise in Squid Eggs: Measurement of Fura-2 Fluorescence

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Sperm break the block to development in unfertilized eggs by somehow inducing a large increase in intracellular free Ca^{2+} ($[Ca^{2+}]_i$). The mechanisms and sources for the increase in $[Ca^{2+}]_i$ have been hypothesized to be different in deuterostome and protostome eggs (1). A release of intracellular Ca^{2+} upon fertilization plays a major role in the regenerative and explosive increase in $[Ca^{2+}]_i$ in deuterostome eggs. By contrast, extracellular Ca^{2+} entry may be more important for the prolonged increase in $[Ca^{2+}]_i$ in protostome eggs. However, solid evidence substantiating this hypothesis in protostome eggs is limited. Squid eggs are unique in their size, mode of development, and phylogenetic position among protostomes (2). We have used these eggs to 1) investigate the existence of voltage-dependent calcium channels in unfertilized egg membranes, 2) establish the methodology of the fura-2 fluorescence measurement, and 3) correlate KCl-induced Ca^{2+} rise with egg activation.

Squid (*Loligo pealei*) were provided by the Marine Resources Department of the Marine Biological Laboratory during July and August. Clear, mature eggs were collected from the oviduct of female squid and washed several times with aerated millipore filtered artificial seawater (ASW). These eggs were put on coverslips, coated with poly-1-lysine, and maintained in ASW. A dual-excitation photometer (Deltascan; Photon Technology Inc.) was combined with the fura-2 technique (3) to measure $[Ca^{2+}]_i$. Eggs were loaded with 10 μM fura-2/AM [the membrane-permeable form, dissolved in dimethyl sulfoxide (DMSO)] for 3 h at room temperature, in the dark. Subsequently, the eggs were thoroughly washed with ASW and then mounted on the stage of an epifluorescence inverted microscope (Nikon diaphot). The fluorescence ratio obtained with two excitation wavelengths, *i.e.*, 350 nm and 380 nm, was used to monitor the relative $[Ca^{2+}]_i$. EGTA (1 mM) was added to nominally Ca^{2+}-free ASW to make Ca^{2+}-free artificial seawater (CaFASW). The appearance of polar bodies 1 h after the addition of either KCl or sperm was taken as indicating that the squid eggs had been activated or fertilized. All experiments were executed with batches of eggs exhibiting less than 6% spontaneous activation.

Intracellular fura-2 loading was reproducible in every batch of squid eggs. High concentration of extracellular K^+ applied to squid eggs maintained in ASW increased $[Ca^{2+}]_i$; two patterns of this effect were observed (Fig. 1). First, rapid increase in $[Ca^{2+}]_i$ induced by 15 mM KCl was seen in eight eggs (fast pattern). The increase in $[Ca^{2+}]_i$ was maximally developed within 1 min and was maintained at this enhanced level for more than 10 min after the addition of KCl. Moreover, this increased $[Ca^{2+}]_i$ did not tend to decay subsequently. In another group of eggs, 15 mM KCl only induced a slow increase in $[Ca^{2+}]_i$ (n = 5) (slow pattern). Here, $[Ca^{2+}]_i$ gradually rose over 30 min after the application of KCl. Only the slow pattern of increase in $[Ca^{2+}]_i$ could be induced by 15 mM KCl in eggs that were exposed to CaFASW. Furthermore, the amplitude of KCl-induced slow increase in $[Ca^{2+}]_i$ in eggs bathed in CaFASW was approximately 37% (n = 3) of that in eggs bathed in ASW. These results suggest that a high concentration of extracellular K^+ can increase $[Ca^{2+}]_i$ by acting on both extracellular and intracellular calcium pools with extracellular pool as the major source. In eggs exposed to ASW without a KCl challenge, neither pattern of $[Ca^{2+}]_i$ change could be observed.

After correcting for the spontaneous activation, we found that 26 ± 6% and 22 ± 6% of eggs, respectively loaded and not loaded with fura-2, were fertilized 60 min after the addition of squid sperm. DMSO alone did not affect the percentage of fertilization of eggs. Morphological observation also revealed that 15 mM

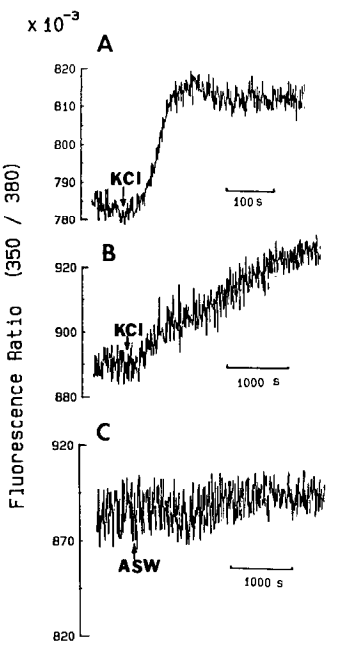

Figure 1. *KCl-induced increase in $[Ca^{2+}]_i$ in squid eggs. The eggs were bathed with 2 ml ASW in Petri dish. A. Addition of KCl at a final concentration of 15 m*M *induced a rapid increase in $[Ca^{2+}]_i$. B. Addition of KCl at a final concentration of 15 m*M *induced a slow increase in $[Ca^{2+}]_i$. C. Addition of 10 μl ASW (control) did not affect $[Ca^{2+}]_i$.*

KCl activated 15.5 ± 0.4% eggs bathed in ASW, which was significantly higher than the percentage of eggs that were activated spontaneously (5.6 ± 0.3%; $P < 0.05$).

While sperm-induced calcium entry has been demonstrated in other protostome eggs, such as those of Urechis, by use of a $^{45}Ca^{2+}$ uptake technique (4), our report is the first one, using the fura-2 technique, to provide the indirect evidence that a voltage-dependent pathway for the extracellular Ca^{2+} entry exists in unfertilized squid eggs. However, it is worth noting that KCl also induced a $[Ca^{2+}]_i$ rise via an extracellular-Ca^{2+} independent mechanism. Furthermore, because fura-2 loading did not interfere with the fertilization process, the methodology of using the fura-2 technique in squid eggs should greatly facilitate further efforts to correlate $[Ca^{2+}]_i$ change with fertilization in squid eggs.

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Differences in Free Calcium Concentration Between Oocytes and Nurse Cells Revealed by Corrected Aequorin Luminescence

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In the ovarian follicles of the moth, *Hyalophora cecropia,* free Ca^{++} is not evenly distributed. Luminescence of microinjected aequorin suggests that internal Ca^{++} activity (Ca_i) is 50% higher in the oocytes than in the attached nurse cells. Each follicle consists of epithelium surrounding an oocyte and seven nurse cells; the latter cells supplying the oocyte with the RNA used in early development. The nurse cells communicate with the oocyte by >30 μm wide intercellular bridges, across which there is an electrical gradient of 2–10 mV. This gradient is such that nurse cells are more electronegative than the oocyte to which they are attached (1–3). The electrical gradient influences the movement of macromolecules through the bridges and thus brings about different equilibrium concentrations in each cell type, dependent on the charge on the molecules (2–5). It now appears that Ca^{++} may contribute either directly or indirectly to this electrical gradient.

Ovaries were dissected from female *Hyalophora cecropia* pupae in the 18th day of the pupal-adult molt. Follicles were maintained in physiological salt solution (PSS) to which 10% cell free blood was added (6). Following removal of the ovariole sheaths, the follicles were treated with collagenase (1 mg/ml PSS) to soften the basement membrane (3). Microinjections were performed using a Leitz micromanipulator and a Medical Systems Corp. PLI-100 microinjector system. Microinjected follicles were viewed with a Zeiss IM-35 microscope coupled to an ultra-sensitive imaging photon detector (7). To visualize the distribution of internal ionic Ca^{++}, 10 n l or less of a 0.62% recombinant aequorin solution, luminescence of which is dependent on free Ca^{++} (8), was injected into either the oocyte or one nurse cell of each follicle. Because the protein may not distribute evenly among the cells, it is essential to know the final distribution of the aequorin. Relative differences in virtual path length (thickness of tissue and relative opacity) through different parts of the follicle also must be considered. To accomplish this, aequorin to which fluorescein had been conjugated was mixed with unconjugated aequorin, and the two forms co-injected. The imaging photon detector was used to acquire signals either from the fluorescence of photoexcited conjugated aequorin, or from the Ca^{++}-induced luminescence of unconjugated aequorin. The acquired photon counts were stored on the Winchester drive of a Compuadd 200 computer.

Analyses of both the fluorescent and the luminescent images were performed by selecting representative 25 × 25-pixel blocks from the background, from the oocyte, and from the nurse cells and tabulating the number of photons/s recorded from each of these areas. After correction for background, oocyte luminescence ÷ nurse cell luminescence × nurse cell fluorescence ÷ oocyte fluorescence adjusts for differences in the concentration of aequorin between the two cell types and also for differences in

Table I

Corrected luminescent ratios between oocytes and nurse cells (N.C.)

Oocyte/N.C. complex number	Oocyte: N.C. corrected luminescent ratio	Oocyte injected	N.C. injected	Time after aequorin inject. (mins.)
July 302 (i)	1.7	+	–	40
July 302 (ii)	4.1	+	–	92
July 304 (i)	1.5	+	–	170
July 304 (ii)	2.3	+	–	180
July 305 (i)	3.8	+	–	206
July 305 (ii)	4.2	+	–	224
July 307*	2.1	+	–	272
July 308*	2.8	+	–	291
July 309*	1.9	+	–	306
July 311	2.4	–	+	250
July 312	6.5	–	+	270
July 313	2.4	–	+	292
July 315	2.2	–	+	335
Avg. Ratios	2.9 ± 0.4	2.7 ± 0.4	3.4 ± 1.1	

* *Unlike the other complexes, these did not undergo a collagenase treatment before microinjection of aequorin.*