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.*

their virtual path lengths. Thus, data were obtained, which gave the distribution of aequorin and also the relative Ca_i corrected for background, path length, and asymmetric aequorin distribution. Observation of follicles began within 40 min after microinjection, and continued either continuously or intermittently for several hours. By the following day, injected cells retained levels of photon emission comparable to those seen at the beginning of their incubation. This continued luminescence demonstrated that the membranes were still competent to exclude external Ca^{++}. Therefore, at least by this criterion, the follicles remained healthy even 24 h after aequorin was introduced into the cytoplasm.

Microinjected aequorin distribution was measured in nine oocyte injected follicles and four nurse cell injected follicles. In the oocyte injected follicles the electronegative aequorin remained more concentrated in the oocyte than in the nurse cells, in agreement with the pattern found previously for other electronegative microinjected proteins (1–3). An additional five follicles, into which only unconjugated aequorin had been microinjected, showed the same pattern. In addition to the four nurse cell injected follicles indicated in Table I, two more follicles received their injections into nurse cell nuclei, where most of the microinjected aequorin remained. This nuclear-bound aequorin revealed a continuing high level of calcium activity. In each of the four nurse cell injected follicles, aequorin had already reached the ooplasm by the time the follicles were examined 40 min after the injection.

As the corrected luminescence of aequorin is around 3 times greater in the oocyte than in the attached nurse cells (Table I), we estimate Ca_i to be 50% higher in the oocyte (9). Because oocytes are less negatively charged when compared to their nurse cells (3), higher Ca_i in the ooplasm can not be explained by Ca^{++} following its' electrochemical gradient. Indeed, the distribution of Ca_i between oocyte and nurse cells revealed by aequorin suggests that Ca^{++} may play a major role in the establishment of the transbridge potential between these two cell types.

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Protease-Binding Activity of *Limulus* α_2-*Macroglobulin*

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α_2-Macroglobulin (α_2M), a major protease-binding protein in the plasma of vertebrates and invertebrates, has been proposed to be the mediator of an evolutionarily conserved immune defense system (1). In vertebrates, binding of proteases by α_2M involves both non-covalent and covalent modes. Non-covalent binding involves a physical folding of α_2M around the protease molecule to "trap" it in the folds of an α_2M "cage" (2). The change in conformation of α_2-macroglobulin necessary for physical entrapment of proteases can be detected by its faster migration during non-denaturing polyacrylamide gel electrophoresis (3). Covalent binding involves the establishment of isopeptide bonds between nucleophilic residues on the protease and the γ-carbonyl of a unique reactive internal thiol ester that becomes activated following proteolysis of α_2M. The covalent binding can be shown by the presence of protease-containing high molecular mass complexes following SDS-polyacrylamide gel electrophoresis (4). It has been proposed that the high molecular mass complexes formed by this mode of covalent binding contain one or several fragments of α_2M linked by the protease molecule (4, 5). The involvement of the thiol ester in covalent binding can be demonstrated by the abolition of binding following pretreatment of α_2M with small amines, such as methylamine, that inactivate the thiol ester. Although tetrameric human α_2M can react successfully with proteases without forming covalent bonds, dimeric and monomeric forms of mammalian α_2M require isopeptide bond formation for binding (6, 7).

The form of α_2-macroglobulin in the blood of the American horseshoe crab (designated LAM for *L*imulus α_2-*m*acroglobulin) reacts with proteases of all major classes to inhibit activity against protein substrates (8, 9) and is organized as a homo-dimer (10). LAM binds one mole of protease per mole of dimer (data not shown). During reaction with proteases, LAM undergoes a major conformational compaction, as evidenced by more rapid mobility in non-denaturing polyacrylamide gel electrophoresis and slower migration during size exclusion chromatography. The extent of compaction is larger than that of human α_2M: human α_2M experienced a 28% increase in mobility in non-denaturing polyacrylamide gel electrophoresis, whereas LAM experienced a 38% increase.

LAM establishes isopeptide bonds following reaction with proteases (Fig. 1). As determined by reducing SDS-PAGE, the subunit of native LAM has a molecular mass of 185 kDa (lane 2). High molecular mass bands of apparent molecular masses 200, 250, and 300-350 kDa were present following reaction with