The Part Played By Inositol Trisphosphate and Calcium in the Propagation of the Fertilization Wave in Sea Urchin Eggs

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Abstract. Sea urchin egg activation at fertilization is progressive, beginning at the point of sperm entry and moving across the egg with a velocity of 5 μm/s. This activation wave (Kacser, H., 1955, *J. Exp. Biol.*, 32:451–467) has been suggested to be the result of a progressive release of calcium from a store within the egg cytoplasm (Jaffe, L. F., 1983, *Dev. Biol.*, 99:265–276). The progressive release of calcium may be due to the production of inositol trisphosphate (InsP₃), a second messenger. We show here that (*a*) a wave of calcium release crosses the *Lytechinus pictus* egg; the peak of the wave travels with a velocity of 5 μm/s; (*b*) microinjection of InsP₃ causes the release of calcium within the egg; (*c*) calcium release (as judged by fertilization envelope elevation) is abolished by prior injection of the calcium chelator EGTA; (*d*) neomycin, an inhibitor of InsP₃ production, does not prevent the release of calcium in response to InsP₃ but does abolish the wave of calcium release; (*e*) the egg cytoplasm rapidly buffers microinjected calcium; (*f*) the calcium concentration required to cause fertilization membrane elevation when microinjected is very similar to that required to stimulate the production of InsP₃ in vitro; and (*g*) the progressive fertilization membrane elevation seen after microinjection of calcium buffers appears to be due to diffusion of the buffer across the egg cytoplasm rather than to the induction of the activation wave. We conclude that InsP₃ diffuses through the egg cytoplasm much more readily than calcium ions and that calcium-stimulated production of InsP₃ and InsP₃-induced calcium release from an internal store can account for the progressive release of calcium at fertilization.

WHEN a sea urchin egg is fertilized, the fertilization envelope rises around the egg progressively, starting from the point of sperm entry (2, 20, 30). The envelope is formed as a result of the exocytosis of cortical secretory granules which lie immediately beneath the plasma membrane of the unfertilized egg (22, 24). These granules undergo exocytosis in vitro in response to micromolar concentrations of calcium ions (11, 13, 40). This has led to the suggestion that the progressive elevation of the fertilization envelope may be due to a progressive rise in intracellular free calcium ion concentration with similar temporal and spatial characteristics (20).

We have shown that microinjection of inositol trisphosphate (InsP₃)[1] into unfertilized sea urchin eggs causes them to raise fertilization envelopes (41). InsP₃ is formed by hydrolysis of a phospholipid, phosphatidylinositol bisphosphate (PtdInsP₂), at fertilization (9). It is thought to act as a second messenger in many cell types by releasing calcium ions from intracellular stores (7).

We have also shown that the production of InsP₃ by isolated egg plasma membranes is stimulated by micromolar calcium concentrations (42). This observation suggests a positive feedback loop of InsP₃ production in sea urchin eggs in which calcium-stimulated production of InsP₃ will lead to further calcium release. We have suggested that this autocatalytic pathway may be responsible for the propagation of the cortical granule exocytosis around the egg at fertilization (41). An observation we made supports this idea: we found that localized injection of InsP₃ into unfertilized eggs caused a progressive elevation of the fertilization envelope which was similar in time course to that caused by sperm and which could not be readily explained in terms of the diffusion of exogenous InsP₃ across the egg.

Two previous sets of observations may be inconsistent with this hypothesis in its simplest form. While a progressive increase in intracellular free calcium has been shown to occur at fertilization in sea urchin eggs, in one species, *Lytechinus variegatus* (5), it is twice as fast as the wave of exocytosis (5, 41). Furthermore, though calcium buffers activate eggs when microinjected, they are reported to do so at concentrations an order of magnitude smaller than those required to stimulate hydrolysis of PtdInsP₂ (19, 42).

This paper reports experiments that investigate these inconsistencies, which may in part be due to species differences and to differences in the estimates of free calcium concentration in calcium-EGTA buffers. We have made all our experiments on the eggs of *Lytechinus pictus* with self-

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1. *Abbreviations used in this paper*: ICM, intracellular medium; InsP₃, inositol (1,4,5)trisphosphate; PtdInsP₂, phosphatidylinositol bisphosphate.

consistent estimates of free calcium concentration. We find that the peak of cytoplasmic calcium concentration crosses the egg at fertilization in a time similar to the time taken for progressive fertilization envelope elevation. We show that microinjection of InsP₃ causes an increase in cytoplasmic free calcium and that the threshold calcium concentration required for egg activation is similar to that required to stimulate PtdInsP₂ hydrolysis and to the concentrations measured in situ at fertilization (25, 30). We test the idea of an autocatalytic cycle of production of InsP₃ and of calcium release by microinjecting neomycin, a drug known to prevent the hydrolysis of PtdInsP₂. We show that egg cytoplasm buffers calcium ions strongly, a result which is consistent with the endogenous, diffusible component of the activation wave being InsP₃.

Materials and Methods

Handling of Gametes

Lytechinus pictus (Pacific Bio-Marine Laboratories Inc., Venice, CA) eggs were obtained by intracoelomic injection of 0.5 M potassium chloride, and egg jelly was removed by passage through Nitex mesh, agitation, and two washes in artificial sea water (450 mM NaCl, 50 mM MgCl₂, 11 mM CaCl₂, 10 mM KCl, 2.5 mM NaHCO₃, 1 mM EDTA, pH 8.0). Eggs were lightly attached to coverslips pretreated with poly-L-lysine (0.01 mg/ml) for microinjection. Eggs were kept at 16°C.

Microinjection Techniques

Micropipettes were formed on a Kopf (David Kopf Instruments, Tujunga, CA) puller and occasionally bevelled using a WPI coarse bevelling disc (Clarke Electromedical, Pangbourne, UK). The pipette-tip diameters were such that a 10-ms pulse of 2,000 kPa pressure ejected a volume of 2.5–5 pl from the pipette. Pressure pulses were delivered to the pipette using a solenoid valve (British Fluidics, Bishop's Stortford, UK) attached to the pipette by 1 m of teflon tubing (0.8-mm i.d., Omnifit, Cambridge, UK). Pressure pulses were delivered to the pipette intermittently (1 Hz) while the pipette was immersed in sea water to prevent contamination of the fluid in the pipette tip by sea water. The micropipette was held in a micromanipulator (Prior, Cambridge, UK) mounted on the stage of a Leitz Diavert microscope.

Measurement of Cytoplasmic Free Calcium Using Aequorin

Lyophilized aequorin (Blinks, Mayo Clinic, Rochester, MN) was dissolved in distilled water and dialyzed against 480 mM NaCl, 20 mM Pipes, 100 µM EGTA, pH 6.7, to give a protein concentration of 20 mg/ml.

Light from aequorin-injected eggs was projected onto the photocathode of an imaging photon detector (ITL Limited, Hastings, UK) using a condenser top lens (1.2 NA Leitz Instruments, Luton, UK). Digitized data from the IPD was processed using an Apple microcomputer and stored on magnetic disk (37).

The aequorin light signal was calibrated in a solution resembling the egg cytoplasm in its composition (intracellular medium [ICM]; 220 mM potassium glutamate, 500 mM glycine, 10 mM sodium chloride, 2.5 mM magnesium chloride, pH 6.7) using calcium-EGTA buffers (10 mM; see below). A calibration curve was drawn relating free calcium concentration to the ratio of peak light output to maximum light output at saturating calcium concentration (1). The cytoplasmic calcium concentration was estimated by determining the ratio of aequorin light emission from the egg to the peak light emission when the egg was lysed at the end of the experiment using Triton

Table I. Microinjection Buffers

Free calcium concentration in calcium buffers			Composition of injection buffers		
Buffer	Calcium/ buffer ratio	Free calcium concentration in ICM	K₂EGTA*	KCl	K-Pipes
		µm	*mM*	*mM*	*mM*
EGTA	0.475	1.3	1	480	20
	0.607	2.7	10	470	20
	0.723	3.8	100	380	20
	0.756	5.0	250	230	20
	0.838	8.0			
	0.915	16			
	0.942	25			
BAPTA	0.30	0.8			
	0.60	1.6			
	0.75	3.2			

* Stoichiometric proportion of K⁺ to EGTA at pH 6.7, 2.2:1 by titration.

X-100. Peak light emission in sea water was 80% of that in the calibration solution; the free calcium values are corrected accordingly. In each experiment, <1% of the aequorin was consumed before lysis.
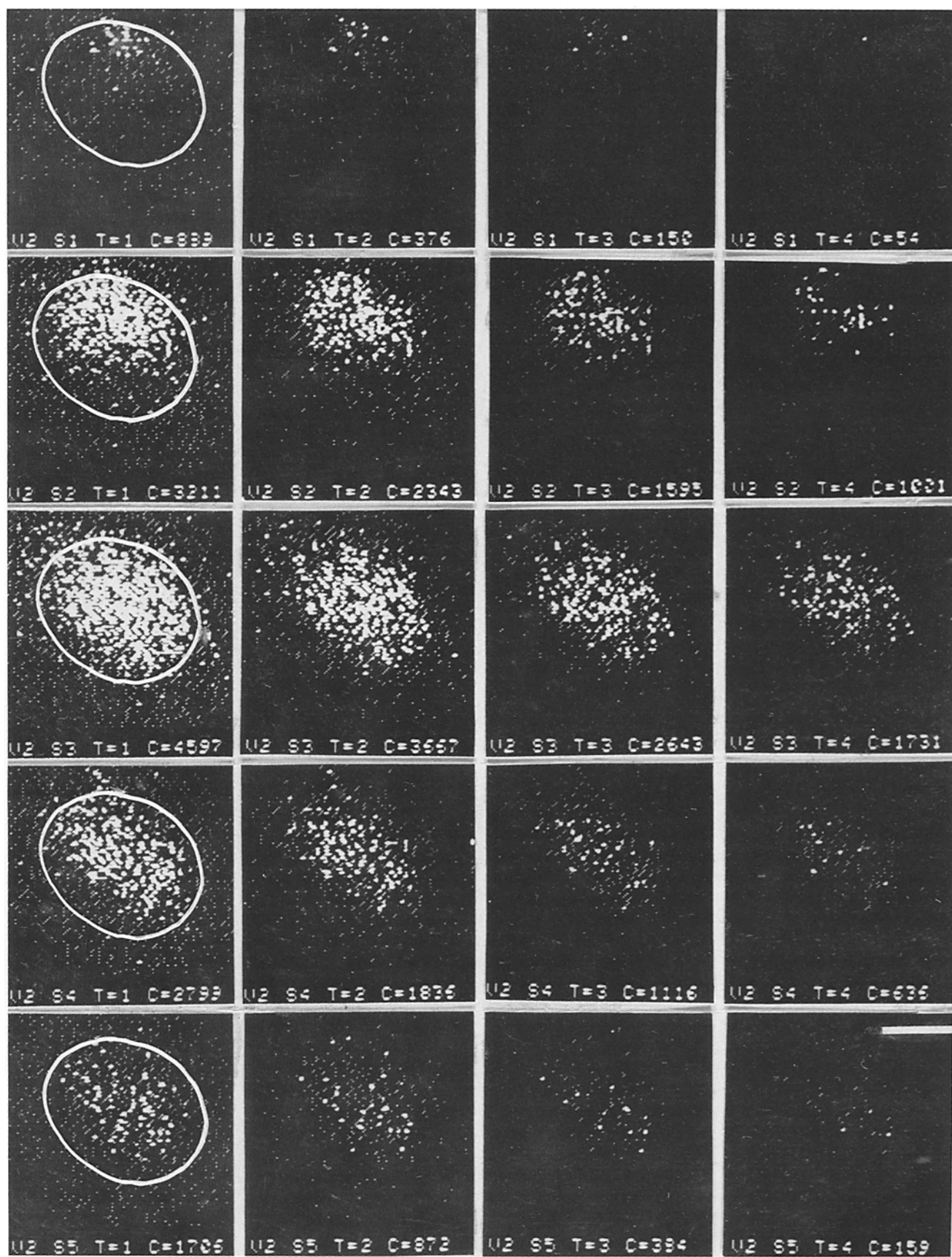
Fluorescent Calcium Indicator Dyes

Fura II (Molecular Probes, Inc., Junction City, OR) was introduced into the egg cytoplasm by microinjection. Excitation of individual eggs was achieved using a mercury lamp and a Leitz Ploemopak epifluorescence attachment on a Leitz Diavert microscope. Emitted light was measured using a photomultiplier (9824B, EMI Electron Tubes Ltd., Hayes, UK). A 350-nm band pass filter (11.2 nm FWHM) and a 380-nm band pass filter (10 nm FWHM) provided exciting light via a 400-nm dichroic mirror. A 490-nm band pass emission filter (7.2 nm FWHM) was used. Data were recorded on a chart recorder and on magnetic tape or disk. Fura II signals were calibrated using the method, equations, binding constants, and calibration solutions of Poenie et al. (25) and the signal from droplets of these solutions placed on the microscope stage.

Calcium Buffers

The free calcium concentration of calcium-EGTA buffers in ICM was calculated using the binding constants of Martell and Smith (23), corrected for the variation of hydrogen ion activity with ionic strength (34). These calculated values are in good agreement with those obtained by measurement after calibrating a calcium ion-sensitive electrode by an absolute method using titration of the calcium-sensitive dye arsenazo III (36). The free calcium concentrations in ICM were additionally verified by using a calcium ion-sensitive electrode and fura II, taking K_{app} for fura II as 0.774 µM (25). The free calcium concentrations of calcium-BAPTA buffers were measured by using a calcium ion-sensitive electrode to compare them with calcium-EGTA buffers. The composition of the calcium buffers we used in given in Table I. They do not contain magnesium ions. The free magnesium ion concentration of egg cytoplasm is estimated to be 3 mM using ion-sensitive microelectrodes (32). We have verified by calculation and with calcium-sensitive electrodes that a free magnesium concentration of 3 mM would not cause us to underestimate the internal calcium concentration by more than a factor of 1.5. Calcium-dependent aequorin light emission is more sensitive to alterations in the magnesium concentration than is fura II (1, 33); comparison of the estimates of cytoplasmic free calcium obtained by each of these methods confirm that the free magnesium concentration is <5 mM. Although the calcium buffers we used for our microinjection experiments

Figure 1. Light emission from an aequorin-injected egg at fertilization. Each of the five rows of images consists of data from one 10-s sampling period. The left column of images displays all pixels in the 128 × 128 array which contain one or more photon counts. The columns to the right display only those pixels with two or more, three or more, or four or more photon counts, respectively. The increase in cytoplasmic calcium occurred equatorially 4 s into the first sampling period shown. Aequorin light emission fills the whole egg (outlined) towards the end of the third 10-s sampling period. Total counts are greatest at the time at which the light emission fills the whole egg (mean free calcium:1.3 µM), but the light emission during the first 10 s indicates that the calcium concentration close to the site of fertilization is 8.0 µM. The detection limit in these experiments was 300–500 nM.

U2 S1 T=1 C=839	U2 S1 T=2 C=376	U2 S1 T=3 C=150	U2 S1 T=4 C=54
U2 S2 T=1 C=3211	U2 S2 T=2 C=2343	U2 S2 T=3 C=1595	U2 S2 T=4 C=1031
U2 S3 T=1 C=4597	U2 S3 T=2 C=3657	U2 S3 T=3 C=2643	U2 S3 T=4 C=1731
U2 S4 T=1 C=2799	U2 S4 T=2 C=1836	U2 S4 T=3 C=1116	U2 S4 T=4 C=636
U2 S5 T=1 C=1706	U2 S5 T=2 C=872	U2 S5 T=3 C=394	U2 S5 T=4 C=159

disperse rapidly when introduced into the cytoplasm (they will be diluted to 10% by cytoplasm in 1 s [14, 41]), the free calcium concentration in buffers of this ionic strength is not known with certainty (29). Measurement of the free calcium concentration of these buffers with the calcium-sensitive photoprotein aequorin indicates that it differs by no more than a factor of 1.5 in the buffers we microinjected. As a final point, although we wish to make it clear that there is an internal consistency in the calibration of calcium-EGTA buffers and in the values of calcium we quote here and in previous papers, we wish to emphasize that our absolute values of free calcium concentration are not intended to imply a precision better than a factor of two.

Results

The Increase in Cytoplasmic Calcium at Fertilization

We measured the progressive increase in cytoplasmic calcium in *Lytechinus pictus* eggs using the calcium-dependent photoprotein aequorin (28) and image intensification techniques.

The light emission of an aequorin-injected egg at fertilization is shown in Fig. 1. The rows of images are formed by summation of the light output over successive 10-s periods. The left column represents the spatial distribution of the light emission. The remaining columns are a measure of the intensity of the light emission at different points within the egg (see legend). The poles of the egg are taken to be at the center of the image of the egg; the equator is in the plane of focus and constitutes the boundary of the disc which represents the egg in the image plane. The top row of images displays the light emission in a 10-s interval during which (at 5 s) an increase in calcium was first detected close to the egg equator. The area of light emission spreads across the egg during the subsequent 10-s period and fills the cytoplasm uniformly after 8 s of the third 10-s period. In the fourth and fifth 10-s period, the free calcium concentration at the antipode appears to fall more rapidly than that at the point of origin; this has also been noted in spatial imaging of eggs injected with fura II (Poenie, M., R. Y. Tsien, R. A. Steinhardt, and J. Alderton, personal communication).

The increase in cytoplasmic free calcium was quantified by establishing the ratio of light emission to peak light output when the egg was treated with a detergent, Triton X-100, at the end of the experiment. The ratio is a steep monotonic function of the calcium concentration (1). Data from the egg shown in Fig. 1 and from five other eggs are given in Table II. In two eggs (1 and 2) light emission began close to the egg equator and filled the egg outline in 25 s. In four other eggs, light emission began more diffusely in an area 15–30 μm away from the equator and filled the egg outline in 10–20 s. Since the time to mean peak light emission was similar for all eggs, we assume eggs 1 and 2 to have been examples of equatorial sperm–egg fusion and interpret the spatial distribution of aequorin light emission in the other cases to be the result of a more polar sperm–egg interaction. In the two cases of presumptive equatorial sperm fusion, the local cytoplasmic free calcium concentration was greatest at the site of sperm–egg interaction; in these two cases, mean peak light emission occurred before the free calcium concentration had reached a peak at the antipode. The calcium transient in all six eggs was of similar duration, rising to a peak in 21.0 ± 0.8 s (mean ± SEM; $n = 6$) and falling to 1.0 μM mean free calcium in 65 ± 2.2 s (mean ± SEM; $n = 5$). If we assume that eggs 3, 5, and 6 are examples of non-equatorial sperm

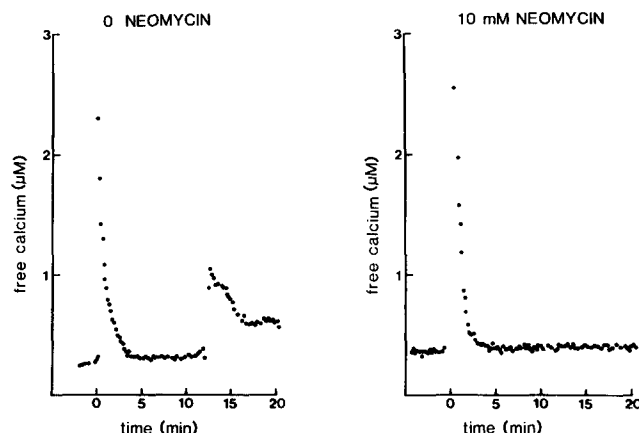*Figure 2.* Free calcium concentration in eggs microinjected with InsP$_3$ and measured using the calcium-sensitive dye fura II (50 μM). The left graph shows the calcium transient in a control egg after injection of 10 μM InsP$_3$ (1% egg volume) at $t = 0$. The right graph shows the calcium transient in an egg that had been microinjected with neomycin to a cytoplasmic concentration of 10 mM 3 min before microinjection of InsP$_3$ at $t = 0$.

penetration, then these eggs too have their highest free calcium at the point of presumptive sperm–egg interaction.

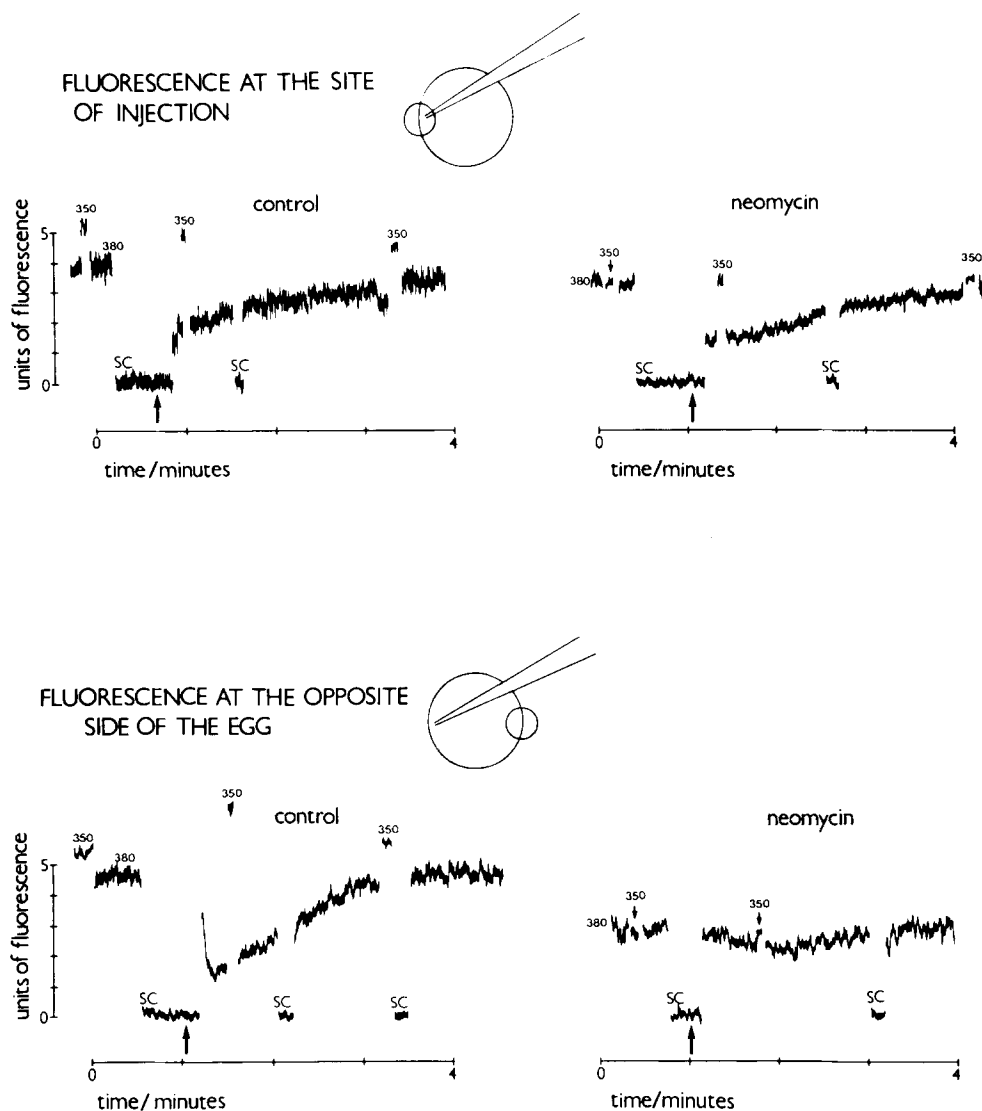### The Effects of Neomycin on InsP$_3$-induced Calcium Release

The antibiotic neomycin prevents the hydrolysis of PtdInsP$_2$ in sea urchin egg plasma membranes (42). If hydrolysis of PtdInsP$_2$ is one component of the activation wave, then one might expect microinjection of neomycin to prevent it. We first made some experiments to determine whether neomycin directly affected InsP$_3$-induced calcium release. We microinjected eggs with the calcium-sensitive dye fura II (25) to measure mean free cytoplasmic calcium concentration, then with neomycin to a cytoplasmic concentration of 10 mM (the concentration of neomycin which inhibits PtdInsP$_2$ hydrolysis and exocytosis in sea urchin egg plasma membranes [42]) and finally injected InsP$_3$ (10 μM, 5 pl; 1% egg volume) into the center of the egg. The cytoplasmic free calcium concentration of neomycin-injected eggs (0.30

Table II. Characteristics of Aequorin Light Emission at Fertilization

Egg	Transit time	Peak free Ca local/mean	Time to mean peak
	s	μM	s
1	25	9.0:1.4	22
2	25	4.0:2.4	20
3	10 < t < 20	—:—	18
4	10 < t < 20	(5.0):2.8	21
5	10 < t < 20	(8.0):1.4	21
6	10 < t < 20	(8.0):1.0	24

The local peak free calcium was determined directly from the aequorin image for eggs 1 and 2. For eggs 4–6, it was assumed that the signal arose from a spherical cap of the same dimensions as that measured for eggs 1 and 2.

FLUORESCENCE AT THE SITE
OF INJECTION

control

350

380

350

350

units of fluorescence

SC

SC

0

4

time/minutes

neomycin

350

380

350

350

SC

SC

0

4

time/minutes

FLUORESCENCE AT THE OPPOSITE
SIDE OF THE EGG

350

control

350

350

380

units of fluorescence

SC

SC

SC

0

4

time/minutes

neomycin

350

350

380

SC

SC

0

4

time/minutes

Figure 3. Local free calcium concentration in eggs microinjected with InsP₃. The fura II (200 μM) signal from a 10-μm diameter area of the egg image was measured as shown in the schematic diagrams. InsP₃ (10 μM, 0.1% egg volume) was microinjected at the egg periphery. We watched the microinjection to be sure that the correct volume had been microinjected close to the periphery, then opened the photomultiplier shutter. SC indicates those periods during the experiment when this shutter was closed. The time of microinjection was recorded by monitoring the signal to the electromagnetic valve which delivered the pressure pulse to the pipette and is marked in the figure by black arrows. 380 and 350 indicate that 380-nm and 350-nm excitation, respectively, was used. Each unit of the scale represents 1.5 mV for the 350-nm excitation and 0.3 mV for 380 nm. The absolute level of the signal depends upon the dye concentration and varied from egg to egg. A decrease in the 380-nm excitation signal indicates an increase in cytoplasmic free calcium. The upper left record shows an example of the fura II signal we measured at the site of InsP₃ injection. The free calcium concentration is already declining at the

site of injection when the shutter is opened 7 s after microinjection. A similar record at the site of InsP₃ microinjection was obtained in egg previously microinjected with neomycin to a cytoplasmic calcium concentration of 10 mM (upper right). In contrast, when the fura II signal was sampled at the antipode in control eggs (bottom left, an egg with a lower fura II concentration) the free calcium concentration had begun to rise at 9 s after microinjection when the shutter was opened and reached a peak at ~20 s before declining slightly faster than at the site of injection. Finally, in neomycin-injected eggs (bottom right) there was no obvious peak of cytoplasmic free calcium concentration at the antipode 20 s after microinjection. Instead the calcium concentration rose slowly to 0.63 ± 0.05 μM (mean ± SEM; n = 8) at 60 s. The traces shown are filtered at 15 Hz.

± 0.09 μM; mean ± SEM; n = 6) before InsP₃ microinjection was slightly higher than that of control eggs (0.22 ± 0.04 μM; mean ± SEM; n = 6). Fig. 2 shows that InsP₃ injection in control eggs causes a cytoplasmic calcium transient. This transient is of similar magnitude and duration to the calcium transient measured at fertilization (Table II and reference 25). Control eggs also consistently showed a second, smaller transient 10–15 min after microinjection of InsP₃. The InsP₃-induced calcium transient in neomycin-injected eggs (2.6 ± 0.2 μM; mean ± SEM; n = 4) was not significantly different from the calcium transient in control eggs injected with InsP₃ (2.6 ± 0.9 μM; mean ± SEM; n = 4). The second, smaller transient was absent in neomycin-treated eggs.

Our previous experiments suggest that when the quantity

of InsP₃ used in the experiment illustrated in Fig. 2 is microinjected, the release of calcium within the egg is due largely to the diffusion of this exogenous InsP₃ through the egg cytoplasm (41). However, these previous experiments also showed that if smaller amounts of InsP₃ are microinjected at the egg periphery, then InsP₃ induces a wave of activation (as judged by cortical granule exocytosis) which has very similar temporal characteristics to the wave of activation induced by sperm. The induction of an activation wave is critically dependent on InsP₃ concentration: 2.5 μM InsP₃ (0.5 pl, 0.1% egg volume) produces only a very local membrane elevation (across <20% of the egg surface, n = 20) whereas 10 μM InsP₃ (0.5 pl, 0.1% egg volume) will trigger a wave of exocytosis which crosses the egg in 25 ± 2 s (mean ± SEM; n = 20).
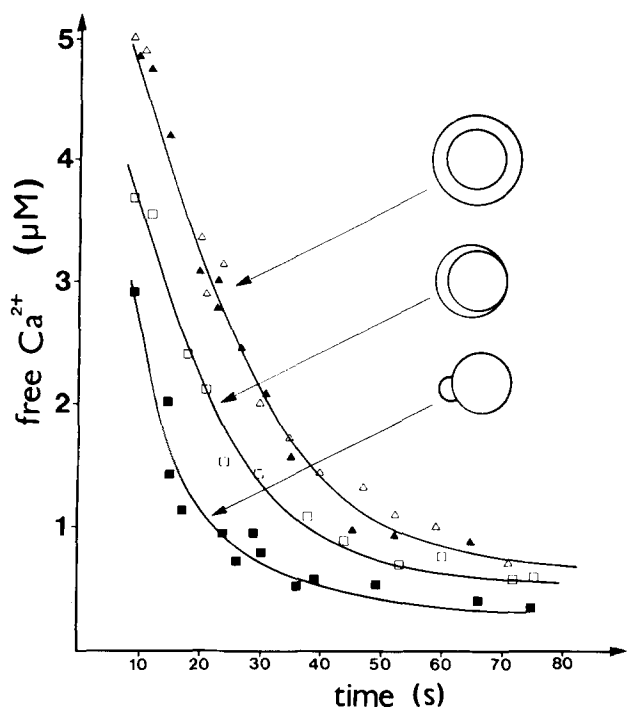
We have measured the free calcium concentration in restricted portions of the egg cytoplasm immediately after injection of these lesser amounts of InsP₃. Fig. 3 shows that if the free calcium concentration is measured at the site of InsP₃ injection (10 μM, 0.5 pl; 0.1% egg volume), then at 6–7 s (the earliest time at which we could measure it) it is already falling and was 1.8 μM at 13 s in the experiment shown. If, however, the free calcium concentration is measured in a restricted portion of cytoplasm at the opposite side of the egg, then the free calcium concentration first begins to increase 7–10 s after microinjection and reaches a peak of 3.7 ± 0.2 μM (mean ± SEM; $n = 7$) after 20.4 ± 0.5 s (mean ± SEM; $n = 7$). These data are consistent with those illustrated in Fig. 1 and suggest that InsP₃ microinjection under these conditions sets off a wave of calcium release whose peak free calcium concentration crosses the egg in 20 s. Fig. 3 also shows the effects of prior injection of 10 mM neomycin on these local alterations in free calcium concentration. Neomycin has no effect on the local free calcium concentration close to the site of microinjection (which was 2.0 and 1.7 μM at 14 and 16 s in two separate experiments) but largely abolishes the peak of free calcium at the antipode (0.34 ± 0.02 μM; $n = 8$ at 20 s after injection). These data suggest that neomycin inhibits the wave of calcium release but that this action is not due to direct inhibition of InsP₃-induced calcium release.

EGTA Prevents InsP₃-induced Activation

We have used the calcium chelator EGTA to estimate the quantity of calcium released when InsP₃ is microinjected into eggs (Table III). If EGTA is microinjected to a final concentration of 1 mM, no activation (elevation of a fertilization envelope) occurs when 1 μM InsP₃ (5 pl; 1% egg volume) is subsequently injected into the egg. On the other hand, a final concentration of 0.1 mM is ineffective. These results indicate that a substantial quantity of calcium is released from intracellular stores by InsP₃ (sufficient to saturate 100 μM cytoplasmic EGTA, that is, at least 100 μmol/liter of egg water) and also suggest that the egg cytoplasm must have a substantial intrinsic buffering capacity if during the release of 100 μmol/liter of egg water of calcium ions after InsP₃ microinjection, the cytoplasmic free calcium is to rise to no more than 3 μM (peak calcium after InsP₃ microinjection: 2.5 ± 0.96; mean ± SEM; $n = 3$; 10 μM, 1% egg volume).

The Calcium-buffering Capacity of the Egg Cytoplasm

We estimated the egg's capacity to buffer calcium by measuring the cytoplasmic calcium concentration with fura II as we injected calcium–EGTA buffers. The cytoplasmic free calcium concentration in unfertilized eggs was 210 ± 40 nM (mean ± SEM; $n = 6$). Fig. 4 indicates that calcium concentra-

Figure 4. Cytoplasmic free calcium concentration immediately after injection of calcium–EGTA buffers. The calcium indicator dye fura II (1 mM in pipette) was injected simultaneously with the calcium buffers at $t = 0$ (1% egg volume): the free calcium concentration computed from the dye signal corresponds to the free calcium concentration in the vicinity of the injected buffer as it diffuses through the egg. All injected solutions had a free calcium concentration of 5 μM (Ca/EGTA:0.756), but different total concentrations of calcium and EGTA. (*Solid square*) 7.56 mM Ca/10 mM EGTA; (*open square*) 37.08 mM Ca/50 mM EGTA; (*solid triangle*) 75.6 mM Ca/100 mM EGTA; (*open triangle*) 189 mM Ca/250 mM EGTA. The data are means of three experiments with each buffer concentration. The cartoons indicate the appearance of the fertilization membrane formed after microinjection.

Table III. Effects of Prior Microinjection of EGTA on the Response to InsP₃

Concentration of EGTA in egg cytoplasm	No activation	Partial activation	Full activation
None	0	0	10
0.1 mM	0	2	8
1 mM	10	0	0

Eggs were injected with 1 μM InsP₃, 5 pl; 1% egg volume.

trations fall very rapidly to 300–500 nM after injection of calcium–EGTA buffers containing 5 μM free calcium. The rate at which the calcium concentration declines depends upon the total amount of calcium injected. It takes 20 s to reach 1 μM when a 5-μM calcium–EGTA buffer containing 7.56 mM calcium is microinjected and 50 s when a 5-μM buffer containing 75.6 mM calcium is microinjected.

Clearly, the time taken to buffer injected calcium may depend in part on the time taken for calcium to diffuse through the egg cytoplasm. 0.01–0.1% of the calcium in these buffers is free in the cytoplasm. The rest is bound to EGTA. It is therefore the diffusion of EGTA species which will determine the time taken for the injected solution to spread uniformly throughout the cytoplasm. The diffusion of EGTA species in situ is hard to detect. We have used instead 6-carboxyfluorescein (376 D, cf. EGTA, 380 D). We measured the fluorescence in a region close to the equator of the egg 25 μm in diameter and microinjected the fluorescent dyes at the opposite side of the egg. The time to 90% fluorescence at the antipode was 40 s for diffusion of 6-carboxyfluorescein. We microinjected a volume (3–5 pl) equivalent to 0.5–1% of the egg volume which means that 40 s after injection, the concentration of a microinjected compound in the egg cytoplasm

will be 0.45–0.9% its concentration in the micropipette. These values are consistent with the rates of calcium buffering shown in Fig. 4 and suggest that the diffusion and buffering of calcium occur at similar rates after microinjection of calcium–EGTA buffers.

Microinjection of Calcium–EGTA Buffers to Determine the Calcium Concentration Required for Exocytosis

The foregoing experiments demonstrate that the interpretation of the effects of calcium buffer injections is not straightforward, but they provide a quantitative background against which the results of calcium buffer injection can be compared.

The microinjection of solutions containing calcium ions and EGTA caused eggs to raise fertilization envelopes. We microinjected a volume equivalent to 1% of the egg volume. Solutions with a Ca/EGTA ratio of 0.756 (5 μM free calcium; see Materials and Methods) caused complete elevation of the fertilization envelope, provided that a sufficient quantity of calcium and EGTA was microinjected. The microinjection of solutions containing 0.756 and 7.56 mM calcium (1 and 10 mM EGTA, 5 μM free calcium) did not cause elevation of complete fertilization envelopes (Table IV). The effects of calcium buffer microinjection appear to depend not only on the free calcium concentration present in the buffer but also on the total concentration of calcium and EGTA, a result that could be predicted from our measurements of the egg's calcium buffering capacity shown in Fig. 4.

Despite the presence of substantial intrinsic cytoplasmic buffering, Table IV shows that we can offer an estimate of the calcium concentration required to trigger fertilization envelope elevation in vivo. Partial elevation of the fertilization envelope beneath the pipette tip occurs with a total calcium concentration of 7.5 mM and a free calcium concentration of 5 μM but not with a total calcium concentration of 47.5 mM and a free concentration of 1.34 μM, indicating that when using 100 mM Ca/EGTA buffers, it is the free rather than the total calcium which determines whether fertilization envelope elevation occurs. The lowest Ca/EGTA ratio which causes partial envelope elevation at this buffer concentration is 0.723. We estimate the free calcium concentration required to bring about exocytosis in *L. pictus* eggs to lie between 2.7 and 3.8 μM (4, 5, 40).

The free calcium concentration of calcium–EGTA buffers is very sensitive to pH. We checked our assumption of an intracellular pH of 6.7 by microinjecting buffers made up with the calcium chelator BAPTA which is less sensitive to pH in

Table IV. Effects of Microinjection of Calcium–EGTA Buffers

Ca	EGTA	Free Ca	No activation	Partial activation	Full activation
mM	mM	μM			
0.75	1	5	10	0	0
7.5	10	5	1	11	0
47.5	100	1.3	21	0	0
60.7	100	2.7	10	5	0
72.3	100	3.8	0	2	32
75.6	100	5.0	0	0	17
75.6*	100*	5.0*	0	14	0

* 0.1% egg volume injected; others, 0.5–1% egg volume injected.

Table V. Effects of Microinjection of Calcium–BAPTA Buffers

Ca	BAPTA*	Free Ca	No activation	Partial activation	Full activation
mM	mM	μM			
30	100	0.8	19	1	0
60	100	1.6	12	0	8
75	100	3.2	3	0	16

* Microinjected in 380 mM KCl, 20 mM K-Pipes, pH 6.7.

the physiological range (33). These results are shown in Table V. The free calcium concentration required to induce cortical granule exocytosis lies between 1.6 and 3.2 μM. This confirms our assumptions in the estimate of the free calcium concentration of Ca/EGTA buffers, once inside the egg.

Microinjection of Calcium–EGTA Buffers and Progressive Fertilization Envelope Elevation

If the mechanism of the progressive fertilization envelope elevation involves the autocatalytic interaction of calcium and InsP₃ as we have suggested, then the process should be set in motion by an increase of either calcium or InsP₃ concentration. When microinjecting calcium buffers or InsP₃, it is important to distinguish between a progressive envelope elevation due to an inherent autocatalytic reaction and that due to simple diffusion of the injected substance. We have shown that InsP₃ microinjection will trigger a stereotypic
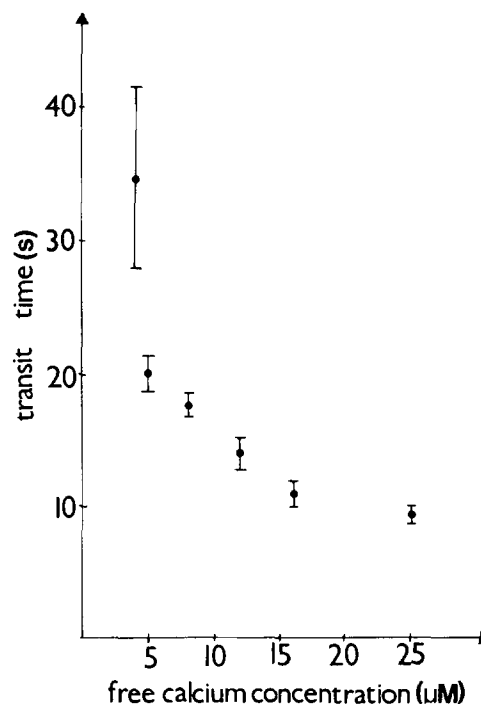

Figure 5. The effect of microinjecting different free calcium concentrations on the transit time for fertilization envelope elevation. Transit time was taken as the time from just noticeable fertilization envelope elevation close to the pipette tip to the time at which the fertilization envelope elevated at the antipode. Microinjected solutions contained 72.3–94.2 mM calcium and 100 mM EGTA (see Table I) and were equivalent to 1% of the egg volume.

progressive fertilization envelope elevation which cannot be attributed to diffusion because the rate of propagation is independent of InsP₃ concentration over a 20-fold range (41). Fig. 5 shows that, although microinjecting calcium–EGTA buffers (≥75.6 mM Ca²⁺; 100 mM EGTA; ≥3.85 μM free calcium; 1% egg volume) causes a progressive elevation of the fertilization envelope, the time between the first signs of elevation close to the site of microinjection (1–2 s after injection) and the time at which elevation occurs at the antipode (the transit time) varies with the free calcium concentration of the microinjected solution. There is no indication in these data of the constant transit time that would be expected if calcium microinjection were triggering an inherent autocatalytic reaction. Rather, the progressive cortical granule exocytosis under these conditions appears to be due to the diffusion of the calcium–EGTA buffers through the cytoplasm.

Discussion

The main purpose of the experiments we have described has been to investigate the relationship between calcium release and the production of InsP₃ at fertilization. We were particularly concerned to obtain data to support or refute the hypothesis (41) that calcium release and InsP₃ production interact to produce the autocatalytic wave which has been suggested to underlie the progressive exocytosis of cortical granules at fertilization (20).

The Progressive Increase in Cytoplasmic Calcium at Fertilization

Our experiments with aequorin indicate that the increase in cytoplasmic free calcium in *Lytechinus pictus* eggs is spatially and temporally inhomogeneous. The cytoplasmic calcium appears to increase first at the presumed point of sperm–egg interaction and the increase appears to spread across the egg over a period of 15–25 s. Our data suggest that the highest local free calcium concentration (though not, of course, the greatest total light output) occurs in the region of cytoplasm in which the disturbance is triggered by the sperm.

The time course of this progressive increase in intracellular calcium is consistent with the idea that it is this process that is responsible for the progressive wave of exocytosis (2, 20). The wave of cortical granule exocytosis crosses the *Lytechinus pictus* egg in 22 s (41). This figure coincides with the transit time of the peak free calcium concentration which we have measured here with aequorin and fura II. Our data differ in this respect from those of Eisen and Reynolds (15) who found a dissociation between the calcium increase and cortical granule exocytosis in *Lytechinus variegatus*, though not in another sea urchin *Arbacia punctulata*. It is possible that non-equatorial sperm penetration may account for the lack of correlation between calcium and exocytosis in *L. variegatus*, since this was a factor which we identified as affecting the apparent transit time in our experiments, though there may be other explanations.

InsP₃ and Calcium Release

Our previous experiments showed that microinjection of InsP₃ led to cortical granule exocytosis and a cytoplasmic

pH change (41). From this we inferred that an increase in cytoplasmic calcium concentration had occurred. Our present experiments demonstrate that this is indeed the case. Microinjection of InsP₃ causes a transient increase of intracellular calcium concentration similar in time course and magnitude to the fertilization calcium transient.

This conclusion is strengthened by the converse experiment in which we prevented the InsP₃-induced propagating response by prior injection of the calcium chelator, EGTA. The concentration of EGTA required to prevent egg activation by InsP₃ is the same as that required to prevent activation by the sperm (44); i.e., a cytoplasmic concentration of 0.5–1 mM. It is clear that InsP₃ activates sea urchin eggs by releasing calcium, presumably from an internal store of the sort that has recently been shown to be sensitive to InsP₃ in vitro (12). We have shown that InsP₃ is produced at fertilization at the time at which the propagated release of calcium is occurring (9). It is reasonable to conclude that InsP₃ is indeed the second messenger responsible for the calcium release at fertilization, though we cannot exclude the possibility that other inositol phosphates may play a part (6, 43).

The Calcium-buffering Capacity of the Egg Cytoplasm

Our experiments indicate that calcium ions introduced into the egg by microinjection are very quickly sequestered, falling to less than micromolar concentrations in <1 min. Two processes appear to contribute to this decrease: diffusion and buffering.

We found that fluorescein, a molecule of similar size to the calcium buffers and indicators we used, diffuses to 90% uniform concentration in 40 s. The substantial decrease in the free calcium concentration which we measured when calcium–EGTA buffers were microinjected together with the calcium indicator dye fura II cannot be attributed to diffusion alone, because the free calcium concentration of a calcium–EGTA buffer is relatively independent of total buffer concentration under these conditions. However, the time course of the decrease in calcium concentration is very similar to the time course of diffusion. One explanation of this might be that the egg pH was more alkaline than the pH of the solution in the micropipette. If the pH of the microinjected solution increased as it spread through the cytoplasm, then free calcium concentration would fall as a result of the marked pH sensitivity of calcium–EGTA buffers. Our experiments with the pH-insensitive calcium–BAPTA buffers demonstrate that this is not a likely explanation. An alternative and reasonable explanation is that the similarity in time course reflects the increasing ability of the egg's calcium buffering systems to reduce free calcium concentration as the cytoplasmic EGTA buffer concentration decreases during diffusion.

In support of this idea, it appears that it is possible to saturate the egg-buffering system transiently with high calcium–EGTA concentrations: at a micropipette EGTA concentration of 100 mM (75.6 mM Ca, 5 μM free calcium), the buffer calcium concentration remains at 5 μM for 10 s (Fig. 3). At the same calcium–EGTA ratio but at lower total buffer concentrations, the egg has reduced the free calcium concentration to 3–4 μM in 10 s. This implies that the rate of calcium uptake lies between 50 and 100 nmol per liter per second.

As the buffer diffuses, free calcium falls more quickly not only because, at a lower buffer concentration, removal of a fixed amount of calcium causes a greater fall in free calcium but also because more cytoplasmic buffering capacity becomes available. These experiments provide an explanation as to why calcium–EGTA buffer injections cause a full fertilization membrane to form only at high buffer concentrations. Full fertilization membranes form when injecting 5 μM free calcium only if the buffer concentration is sufficient to ensure that the free calcium concentration does not fall below 1–2 μM in the time taken for the buffer to diffuse across the egg.

The Free Calcium Concentration Required to Cause Exocytosis

Since buffers of lower total calcium but higher free calcium than ineffective 100 mM calcium–EGTA buffers (injected at 1% egg volume) cause exocytosis to occur close to the pipette tip, we can infer that calcium sequestration does not substantially alter the free calcium concentration of 100 mM calcium–EGTA buffers close to the site of injection. This inference is confirmed by the experiments in which we used fura II to measure the free calcium in buffers during microinjection: as we pointed out above, using 100 mM buffers, the free calcium concentration in the buffer remains at 5 μM for 9–10 s. Calcium sequestration cannot explain the discrepancy between our estimate of the calcium requirement for exocytosis of the cortical granules (2–3 μM free calcium) and the previously published estimate (0.2–0.3 μM for *Hemicentrotus pulcherrimus* [19]). Our data suggest that this discrepancy may have arisen because the authors of this previous study made an overestimate in their assumption of the cytoplasmic pH of the unfertilized egg and an underestimate of the egg cytoplasmic pH buffering capacity, which is equivalent to ten times the buffering capacity of the solutions that both we and they microinjected (18, 26). We calculate from Hamaguchi and Hiramoto's data (19) a value of 1.5 μM for the free calcium concentration required to cause exocytosis in *Hemicentrotus pulcherrimus* eggs, using our binding constants and a pH of 6.7 (27).

Our estimate of the free calcium concentration required to cause exocytosis in situ compares well with the free calcium concentration required to cause cortical granule exocytosis in vitro (3 μM [40]). It is also identical to the calcium concentration required to cause half-maximal activation of phosphatidylinositol bisphosphate phosphodiesterase in isolated egg plasma membrane (42).

The Mechanism of Fertilization Wave

The experiments we report here and others (8, 16) support the idea that the response which propagates through the sea urchin egg cytoplasm at fertilization (21, 31) is due to a progressive release of calcium from an intracellular store. To call this progressive calcium release a wave is perhaps imprecise, since it more closely resembles a wavefront and, as our aequorin images suggest, may be inherently decremental (2). However, the term is widely used. Our suggestion is that this wave of calcium release arises from the mutually reinforcing release of calcium and InsP₃ that comes about because InsP₃ causes calcium release (12, 41) and calcium causes InsP₃ production by stimulating phosphatidylinositol bisphosphate phosphodiesterase (41, 42).

This idea is supported by our observation that neomycin, an inhibitor of PtdInsP₂ hydrolysis (17), prevents the wave of calcium release set off by local injection of InsP₃ without directly inhibiting InsP₃-induced calcium release. The concentration of neomycin required is the concentration we have shown to inhibit PtdInsP₂ hydrolysis in sea urchin egg plasma membranes (41). The inhibition of both PtdInsP₂ hydrolysis and the calcium wave appears to be due to some other mechanism than specific binding to PtdInsP₂ (MacLaughlin, S. J., and M. Whitaker, unpublished observations).

Our results with neomycin suggest that calcium-dependent hydrolysis of PtdInsP₂ is occurring during the activation wave. Why then do we find no evidence of a wave-like response to calcium microinjection? Our conclusion, based on the observations set out below, is that the buffering capacity of the egg cytoplasm is such that calcium ions have a markedly reduced mobility compared to free solution. The buffering capacity of sea urchin egg cytoplasm is comparable to that of squid axoplasm, in which calcium diffuses at a rate at least 50 times slower than in free solution (3), in contrast to InsP₃, which we have shown to diffuse readily (41).

The activation wave induced at fertilization or by InsP₃ microinjection is abolished by prior microinjection of EGTA to a final cytoplasmic concentration of 0.5–1 mM (Table III, reference 44, and our unpublished data). Calcium buffers that completely activate eggs contain comparable amounts of free EGTA and should thus prevent the activation wave from occurring. In other words, as the calcium–EGTA concentration in the micropipette is increased to overcome the egg's intrinsic buffering capacity, so the extrinsic buffer (EGTA) prevents any calcium release which occurs within the egg from substantially affecting the free calcium concentration. The solution to this problem would seem to be to dispense with calcium buffers and inject calcium chloride solutions. However, calcium chloride solutions of up to 1-mM pipette concentration are, as one would predict from the data of Table IV, ineffective, never producing complete egg activation (our unpublished observations; above this pipette concentration the cytoplasm coagulates around the injected solution and it fails to disperse). Our data can therefore account for the failure of calcium and calcium–EGTA buffer injections to trigger an activation wave and for the finding that local application of the calcium ionophore, A23187, to eggs leads to nonpropagating responses (10, 38). They do nonetheless raise the question of how, given the strong buffering capacity of egg cytoplasm, InsP₃ or, indeed, the fertilizing sperm, is able to initiate a propagated release of calcium.

The propagating response to InsP₃ has a well-defined threshold, evidence of its all-or-none properties: below a critical InsP₃ concentration (2.5 μM < InsP₃ < 5 μM) partial fertilization envelope elevation occurs and no activation wave is triggered. If certain calcium microinjections lead to partial fertilization membrane elevation, this cannot be construed as indicating that calcium plays no part in an autocatalytic mechanism. The striking difference between calcium and InsP₃ is the ease with which they can diffuse through the egg cytoplasm. It is this difference which suggests that they are unequal partners in the activation wave. In this, sea urchin eggs appear to differ from *Xenopus laevis* oocytes in which it has been suggested that calcium ions are a diffusible component in a calcium wave (8). Calcium-dependent calcium release seems a less likely mechanism in sea urchin

eggs than in *X. laevis* oocytes because the activation wave in sea urchin eggs cannot be induced by microinjection of unbuffered calcium, in contrast to the wave of calcium release in *X. laevis* oocytes. It appears from our aequorin data that the cytoplasmic free calcium concentration is greatest at the beginning of the activation wave, close to the site of sperm egg interaction, and we can infer that the fertilizing sperm must first cause an increase in $InsP_3$ within the egg. It is possible that a sperm receptor coupled to phosphatidylinositol bisphosphate phosphodiesterase is responsible for the initial increase in $InsP_3$ (35) but other mechanisms are as likely (39, 41).

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