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Sperm-Induced Currents at Fertilization in Sea Urchin Eggs Injected with EGTA and Neomycin

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Membrane currents were measured in single voltage-clamped sea urchin eggs (*Lytechinus pictus* and *Lytechinus variegatus*) that were injected with either EGTA or neomycin and inseminated. Although egg activation and the fertilization calcium wave were prevented by injection of either of these compounds, sperm attached and still elicited inward currents. Sperm-induced currents in EGTA-injected eggs had an abrupt onset, quickly reached a maximum, and then slowly declined in amplitude. Sperm incorporation occurred readily in EGTA-injected eggs. Similar results were obtained with another calcium chelator, BAPTA. In neomycin-injected eggs, sperm-induced currents generally had an abrupt onset and, in contrast to EGTA-injected eggs, the currents usually cut off rapidly. Sperm failed to enter the neomycin-injected eggs and the duration of sperm-induced currents in neomycin-injected eggs was markedly dependent upon the voltage-clamp holding potential, with shorter duration currents occurring at -70 than at -20 mV. The lability of the initial interaction between sperm and egg at negative holding potentials may explain why activation often fails when the egg membrane is voltage clamped at these potentials (Lynn *et al.*, *Dev. Biol.* 128, 305-323, 1988). © 1992 Academic Press, Inc.

INTRODUCTION

The activation of a sea urchin egg at fertilization occurs as the result of a wave of internal calcium release that crosses the egg from the point of sperm-egg contact (Whitaker and Steinhardt, 1982; Eisen *et al.*, 1984; Swann and Whitaker, 1986). This wave of calcium release causes a corresponding wave of exocytosis that is referred to as the fertilization wave (Jaffe, 1983). The calcium wave is thought to be the result of a wave of phosphatidylinositol bisphosphate (PtdInsP₂) hydrolysis that generates the calcium releasing second messenger inositol 1,4,5-trisphosphate (InsP₃) (Turner *et al.*, 1984; Whitaker and Irvine, 1984; Ciapa and Whitaker, 1985; Swann and Whitaker, 1986; Crossley *et al.*, 1988). It is likely that the calcium wave is triggered by an initial local increase in InsP₃ or calcium because small, localized injections of InsP₃ can trigger an autocatalytic wave indistinguishable from the calcium wave at fertilization (Whitaker and Irvine, 1984).

There is a distinct delay period of about 15-30 sec between sperm-egg binding and the onset of the fertilization wave (Allen and Griffin, 1958; Whitaker and Steinhardt, 1982). It is not yet clear what happens during this latent period, although it must represent a gradual process that culminates in the triggering of the autocatalytic calcium wave. The sperm may act through a surface receptor coupled to a GTP-binding protein that stimulates PtdInsP₂ production (Turner *et al.*, 1984, 1986). Alternatively, it has been suggested that the sperm triggers a calcium wave by introducing a soluble factor into the ooplasm when sperm and egg fuse (Dale *et al.*, 1985; Swann and Whitaker, 1990; Whitaker and Crossley, 1990; Crossley *et al.*, 1991).

A precise estimate of the duration of the latent period at fertilization in sea urchin eggs has been made by measuring the changes in egg membrane potential or membrane current during sperm-egg interaction (Chambers and de Armendi, 1979; Schatten and Hulser, 1983; Eisen *et al.*, 1984; Shen and Steinhardt, 1984; Longo *et al.*, 1986). The initial electrical event at fertilization occurs 1-3 sec after sperm-egg contact. There is an abrupt increase (I_{on}) in inward current (the phase 1 current onset) that causes the egg membrane to depolarize from around -70 mV and fire an action potential (Lynn *et al.*, 1988; Jaffe, 1976). The phase 1 current has been suggested to originate from channels in the sperm plasma membrane and to be transmitted to the egg

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² Abbreviations used: BAPTA, bis(*o*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β -aminoethyl ether) *N,N,N',N'*-tetraacetic acid; GTP, guanosine triphosphate; InsP₃, inositol 1,4,5-trisphosphate; PtdInsP₂, phosphatidylinositol bisphosphate; Taps, tris(hydroxymethyl)methyleneaminosulfonic acid.

through a conducting pore (McCulloh and Chambers, 1992). Once the phase 1 current is established, a secondary inward current (the phase 2 current) is seen, simultaneous with the fertilization wave (Lynn *et al.*, 1988). The phase 2 current is thought to be caused by the increase in Ca_i in the egg (Whitaker and Steinhardt, 1982; David *et al.*, 1988; McCulloh and Chambers, 1991). The latent period at fertilization in sea urchins is equivalent to the delay between the start of the initial inward current, at I_{on} , and the start of the phase 2 current (Whitaker and Crossley, 1990).

In this paper we report voltage-clamp experiments on sea urchin eggs of *Lytechinus pictus* and *Lytechinus variegatus*. We have examined the effects of two inhibitors of the PtdInsP₂/Ca²⁺ signaling system on phases 1 and 2 of the sperm-induced inward current. Eggs were injected with either EGTA, which chelates calcium ions, or neomycin which inhibits polyphosphoinositide turnover. Although both of these agents block the generation of the fertilization calcium wave (Zucker and Steinhardt, 1978; Swann and Whitaker, 1986), we found that sperm still cause inward currents in eggs that were injected with these compounds: the two compounds effectively extend the latent period, allowing us to investigate the early stages of sperm-egg interaction. Our data suggest that, if an aqueous pore forms between sperm and egg during the latent period, it is initially labile and reversible. The lifetime of the sperm-egg interaction is reduced as the membrane is held more negative. The increased lability of the putative aqueous pore at more negative potentials could explain why fertilized eggs held at more negative membrane potentials are less likely to activate (Lynn and Chambers, 1984; Chambers and McCulloh, 1990). Some of the results in this paper have appeared in a preliminary form (Swann *et al.*, 1987; Whitaker *et al.*, 1989).

MATERIALS AND METHODS

Handling of Gametes

Eggs of the sea urchin *L. pictus* (Pacific Biomarine Laboratories, Venice, CA) or *L. variegatus* were obtained by injecting females with 0.05 ml of 0.5 M KCl. For *L. pictus* eggs the jelly was removed by passing them through a nitex mesh and then swirling them in artificial seawater (450 mM NaCl, 50 mM MgCl₂, 11 mM CaCl₂, 10 mM KCl, 2.5 mM NaHCO₃, 1 mM EDTA, pH 8). *L. variegatus* eggs were dejellied by washing them several times in natural seawater containing 10 mM Taps (tris[hydroxymethyl]methyleneaminosulfonic acid) adjusted to pH 8.3; further details are given in Longo *et al.* (1986). *L. pictus* eggs were attached to polylysine coated coverslips (0.01 mg/ml) and kept at 16–18°C. The *L. variegatus* eggs that attached spontaneously to untreated

plastic dishes were maintained at 22–23°C. Sperm from the homologous species of sea urchin was collected "dry" from *L. pictus* males injected with KCl in a similar manner to females, or by removal of testes from *L. variegatus* males. This was kept at 4°C until dilution into a small volume of seawater that was then used to inseminate the eggs. Sperm densities were from 10⁴–10⁶ per milliliter at a distance of 1 mm from the egg, such that between 1 and 10 sperm attached to the egg surface within 10 min of insemination.

Electrophysiological Recordings

Eggs were impaled with glass microelectrodes filled with 3 M KCl or 0.5 M potassium citrate (40–80 MΩ resistance) or 0.5 M K₂SO₄ (20–30 MΩ). *L. pictus* eggs were voltage clamped using a single electrode and a switched clamp amplifier based on the design of Wilson and Goldner (1975). *L. variegatus* eggs were voltage clamped with a single electrode system of the Dagan Corporation (Model 81001-1). Eggs were first impaled and a stable recording obtained using small dc current conditions. They were then microinjected with a second micropipet which was only briefly inserted into the egg. After removing the injecting pipet eggs were voltage clamped at the required potential (David *et al.*, 1988) and inseminated. Further details of the impalement and recording procedure are given in Lynn *et al.* (1988) and Longo *et al.* (1986). Only eggs that showed action potentials before injection and that could be clamped at –70 mV with less than 500 pA of holding current were used. The negative capacitance, phase, and gain of the voltage clamp were adjusted to the optimum by using a voltage step (David *et al.*, 1988) before insemination. Membrane conductance was measured using sequential incremental voltage command steps from the holding potential, as in David *et al.* (1988). Currents and voltages were recorded on tape (Racal 4DS) and on a chart recorder, with clamping currents filtered at 0.3 kHz.

Microinjection

Eggs were microinjected with solutions containing either 100 mM EGTA (plus approx. 300 mM KCl, 20 mM Pipes), 500 mM EGTA, 20 mM Pipes, 0.9 M BAPTA, or 0.5 M neomycin adjusted to pH 6.7 with KOH. A pulse pressure system was used to inject eggs with 2–10 pl of solution (Swann and Whitaker, 1986). Concentrations quoted in this paper are calculated final concentrations in the egg cytoplasm unless stated otherwise. The egg volume was taken as 500 pl.

Sperm Fusion/Incorporation Assay

The incorporation of the sperm into eggs was assessed using the technique described by Hinkley *et al.* (1986).

Dejellied eggs were incubated in a solution of H33342 in artificial seawater for 30–60 min. They were then washed free of excess dye by centrifuging and resuspending in normal artificial seawater to reduce the H33342 concentration to less than 10^6 times the loading concentration. Eggs were then attached to coverslips for microinjection of either EGTA or neomycin. Eggs were inseminated 5–10 min after microinjection and then 10 min later they were scored, in the presence of attached sperm, for fluorescent sperm which were counted by viewing eggs on the stage of an epifluorescence Leitz diavert microscope under uv light. In some cases eggs were fixed with 1.5% glutaraldehyde 5–10 min after insemination.

Measurement of Intracellular Free Calcium Concentration

Intracellular free calcium concentration (Ca_i) was measured using the fluorescent indicator dye Fura 2 (Swann and Whitaker, 1986) and UMANS software (C. Regan, Bio-Rad, Hemel Hempstead, UK). In some experiments, the fluorescence signal and clamp current were measured simultaneously and stored using the UMANS system.

Sources of Reagents

All chemicals were of Analar grade and obtained from BDH Limited (Poole, Dorset, UK) except Taps, neomycin, H33342, and EGTA (Sigma Chemical Co. Ltd., Poole, Dorset, UK).

RESULTS

Sperm Entry and Activation in Eggs Injected with EGTA or Neomycin

No obvious difference was noted in the attachment of sperm to eggs injected with EGTA or neomycin, compared to uninjected controls. Inward currents were seen in voltage-clamped eggs after sperm attachment in either species of sea urchin egg injected with either 5–10 mM neomycin or 0.5–5 mM EGTA. However, these concentrations of neomycin or EGTA completely blocked activation as judged by a lack of fertilization envelope elevation (Fig. 1), nuclear envelope breakdown, and first cleavage. A difference between the two inhibitors was observed at the level of sperm entry.

Sperm readily entered EGTA-injected eggs and the fertilization cones were always bigger than normal. In several experiments in eggs clamped at -20 mV it could be clearly seen that the attached sperm's tail stopped beating several seconds after the onset of the inward currents. In *L. variegatus* eggs voltage clamped at -20

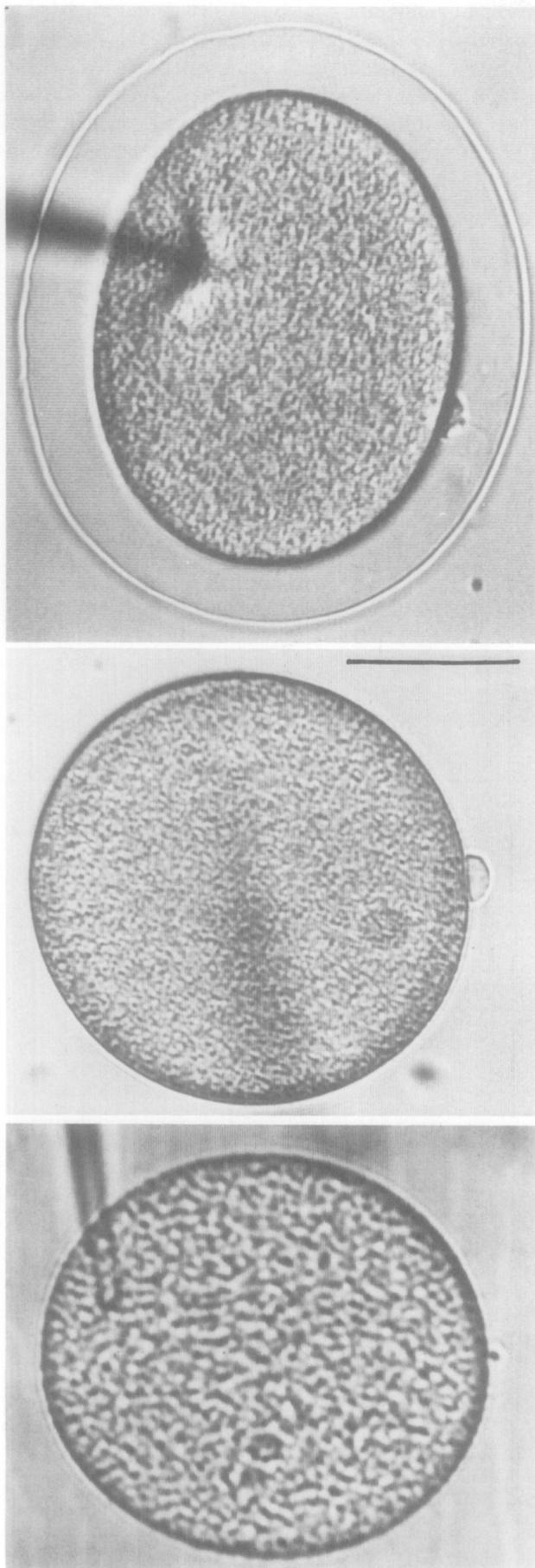
mV, large fertilization cones formed, within which the sperm could frequently be observed (Fig. 1, center). To confirm that EGTA does not block sperm incorporation we tested for the presence of sperm in EGTA-injected eggs (not voltage clamped) using the Hoechst dye transfer assay (Hinkley *et al.*, 1986). Table 1 shows that when *L. pictus* eggs were injected with EGTA (1–2 mM) and inseminated, several sperm incorporated into each egg.

Although sperm readily attached to the surface of the voltage-clamped neomycin-injected eggs and caused inward currents the sperm did not enter the eggs (Fig. 1, right). Occasionally, in *L. variegatus* eggs, sufficiently low sperm densities were achieved in the egg's immediate vicinity that only one sperm attached and an inward current occurred. In these (4/12) cases, the attached sperm was identified as the sperm that caused the current step. In contrast to what we observed in EGTA-injected eggs, in neomycin-injected eggs the sperm tail did not stop beating throughout the occurrence of the current step. Immediately after the sperm-induced current decreased the attached sperm frequently detached from the egg and, on occasion, swam away. These observations indicated to us that the sperm were not incorporated into the egg. To confirm that the sperm did not penetrate eggs injected with neomycin we also assayed sperm incorporation with the Hoechst dye transfer assay. In all cases where eggs (not voltage clamped) were injected with 5–10 mM neomycin and inseminated we could not detect fluorescent sperm within the ooplasm (Table 1). Nor were fluorescent sperm seen with the H33342 assay when neomycin-injected eggs were fixed shortly after insemination and observed 30 min later (Table 1).

The Fertilization Calcium Transient Is Suppressed in both EGTA- and Neomycin-Injected Eggs

The relation between the fertilization current and the fertilization calcium transient in control eggs is illustrated by a representative experiment in Fig. 2A. The fertilization current (I_f) precedes the increase in intracellular free calcium concentration (Ca_i). The time that elapses between the onset of the current and the onset of the Ca_i increase is the latent period. Figure 2A also shows the two phases of the fertilization current (Lynn *et al.*, 1988). The phase 1 current has a rapid onset (I_{on}) and increases to a shoulder (I_{sh}). The phase 2 current supervenes at the shoulder and increases to a maximum (I_{peak}). Figure 2A shows the relation between Ca_i , I_{sh} , and I_{peak} : I_{sh} is seen 10–15 sec after the onset of the Ca_i increase (when mean Ca_i is 0.8–1 μM : Table 2); I_{peak} coincides with the peak of the fertilization calcium transient.

In EGTA- and neomycin-injected eggs (not voltage



clamped), the fertilization calcium transient is suppressed. No detectable Ca_i increase is observed when a sperm attaches; in EGTA-injected eggs, a fertilization cone is observed (Fig. 2B shows one of three representative experiments for each agent).

In Voltage-Clamped Eggs Injected with EGTA Sperm Cause Inward Currents with an Abrupt Onset and Gradual Turnoff

Figures 3A and 3B show that both *L. pictus* and *L. variegatus* eggs voltage clamped at -20 mV display a similar pattern of inward currents after insemination when injected with EGTA solutions (0.5 – 2 mM). The inward currents observed after a single sperm had contacted the egg surface showed an abrupt onset (I_{on}), which slowly increased to a maximum, I_m , at $t = 22.2 \pm 2.1$ sec (*L. variegatus*; mean and SEM; $n = 8$). Following I_m , the inward current decreased until the preinsemination value was attained after about 2 min. The mean amplitude of the currents at I_{on} and at I_m were somewhat smaller in *L. pictus* than in *L. variegatus* at corresponding clamped membrane potentials (Table 3 and Fig. 3; but compare Table 4). The differences were most marked in this series of experiments and may have been due, in part, to differences in temperature or simply species. The current durations were not significantly different (Fig. 7). Figure 3C shows the same type of prolonged inward current in a *L. variegatus* egg injected with EGTA, then clamped at -70 mV, in which sperm entry was observed to occur. The shape and magnitude of the inward current during the first few seconds bear a close resemblance to the initial current recorded during attachment of sperm to control eggs (Fig. 2A), whether voltage clamped at membrane potentials permissive or nonpermissive for sperm entry (Lynn *et al.*, 1988; Longo *et al.*, 1986; David *et al.*, 1988). Following I_m (attained at 22.1 ± 1.2 sec, $n = 12$ in *L. variegatus*), the currents in EGTA-injected eggs steadily decreased, returning to preinsemination levels after approximately 2 min, whether the eggs were voltage clamped at -20 or -70 mV, provided sperm entry occurred.

There were, however, three marked differences between these current records and those seen in normally fertilizing eggs (Lynn *et al.*, 1988). (1) The most marked difference was the absence of phase 2 of the inward

FIG. 1. Photomicrographs of control (top), EGTA-injected (center), and neomycin-injected (bottom) eggs after insemination. Fertilization cones are visible at 4 o'clock in the control and at 3 o'clock in the EGTA-injected egg. No cones are seen in neomycin-injected eggs, but the attached sperm which caused the inward current is shown at 3 o'clock, 1 min after I_{on} . The EGTA- and neomycin-injected eggs lack a fertilization envelope. The bar indicates $50 \mu\text{m}$. *L. variegatus*.

TABLE 1
THE EFFECTS OF MICROINJECTION OF EGTA AND NEOMYCIN
ON THE TRANSFER OF H33342 BETWEEN SPERM AND EGG

Eggs	Mean number of fluorescent sperm in each egg
Controls (not injected)	1.00 (± 0.00 , $n = 20$)
Injected with KCl	1.46 (± 0.18 , $n = 13$)
10 mM neomycin	0.00 (± 0 , $n = 19$)
10 mM neomycin (fixed eggs)	0.0 (± 0 , $n = 5$)
1 mM EGTA	5.3 (± 0.78 , $n = 24$)

Note. The sperm density was about 10^6 per ml for all these experiments. Sperm counts were taken 5–10 min after insemination. None of the eggs injected with neomycin or EGTA had fertilization envelopes. The numbers in parentheses represent SEM, n = number of eggs observed.

current (no I_{peak} was detectable), the phase that correlates with the fertilization Ca_i transient and activation of the egg. (2) There was no slowly developing outward current in EGTA-injected eggs clamped at -20 mV. (3) In some experiments in which a number of sperm found their way to the immediate vicinity of the egg, several sperm contacted the egg surface in succession, due to the lack of fertilization envelope formation. In these cases a distinctive inward current and I_{on} were associated with each sperm (not shown). In several experiments in which a second successful sperm attachment occurred 1–2 sec after the first I_{on} , the inward current at I_{m} was about twice the amplitude of I_{m} after single sperm attachment; summation of this sort is a characteristic of the phase 1 current (Lynn *et al.*, 1988).

We compared the efficacy of EGTA in abolishing the phase 2 fertilization current (I_{peak}) with that of BAPTA, a related calcium chelator that has more rapid chelation kinetics (Marty and Neher, 1985). In 80 separate microinjection experiments using various concentrations of BAPTA and EGTA, phase 2 (as a separate identifiable waveform) and fertilization envelope elevation in *L. variegatus* were fully suppressed at intracellular EGTA concentrations of 0.5 mM and higher, and at BAPTA concentrations of 0.7 mM and higher. Phase 2 and fertilization envelope elevation were fully or only partially suppressed at EGTA concentrations of 0.3–0.5 mM and at BAPTA concentrations of 0.3–0.7 mM. The effective threshold concentrations for these two calcium chelators are thus very similar and also similar to the EGTA concentration reported by Zucker and Steinhardt (1978) as necessary to suppress the fertilization wave in sea urchin eggs.

Microinjecting BAPTA to a higher cytoplasmic concentration of 5 mM resulted in sperm-induced currents identical in form to those seen after injection of 1 mM EGTA and described above (Fig. 4). The magnitude of

the initial rapid current step (I_{on}) was not significantly different from same-day controls in eggs injected with 5 mM BAPTA (see Table 4). At the time at which a Ca_i increase is just detectable in control eggs (t_{ca}), the inward current in BAPTA-injected eggs is not significantly different from controls. At t_{sh} , the time at which

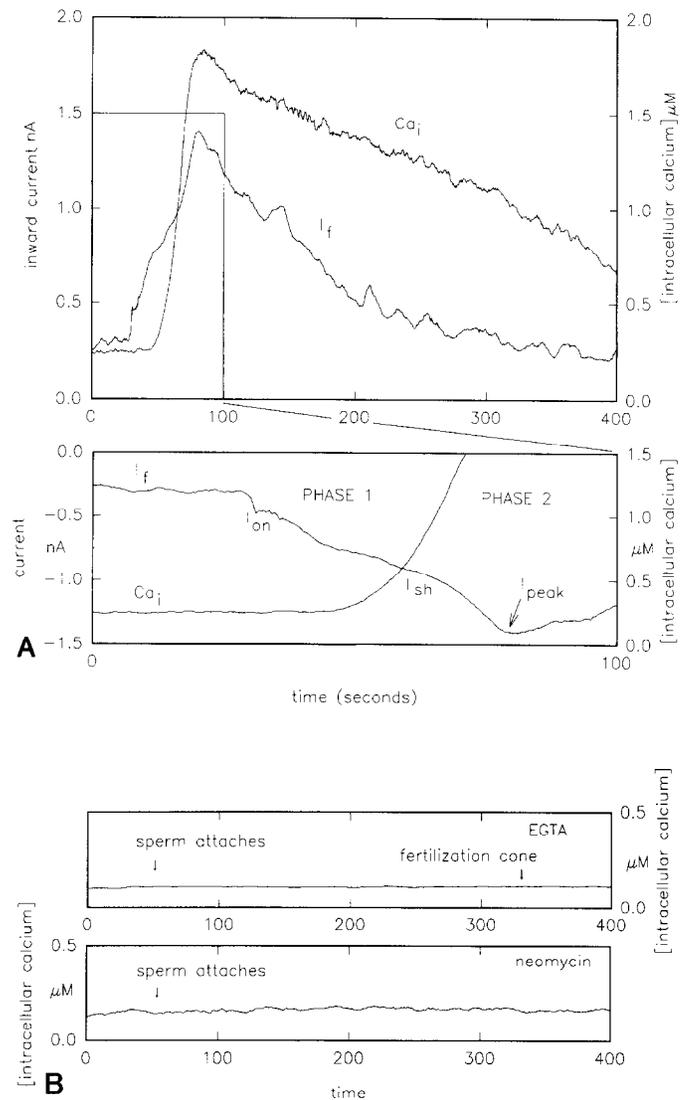


FIG. 2. Intracellular free calcium concentration after insemination of control and EGTA- and neomycin-injected eggs. (A) Simultaneous measurement of Ca_i and membrane current in a control egg voltage-clamped at -50 mV and inseminated at $t = 0$. The onset of the fertilization current (I_f) precedes the Ca_i increase. The peak of the fertilization current coincides with the Ca_i peak. The fertilization current comprises a rapid onset (I_{on}), followed by a more gradual increase to an inflection (I_{sh}). Phase 1 is the sperm-induced current that is measured during the latent period. The inflection marks the appearance of phase 2, the calcium-dependent component of the fertilization current. A representative experiment is shown (see Table 2). (B) Ca_i in EGTA-injected (1 mM) and neomycin-injected (7 mM) eggs after insemination. Sperm attachment and the appearance of a fully formed fertilization cone are indicated by the arrows. One of three similar experiments is shown in each case. *L. pictus*.

TABLE 2
PARAMETERS OF THE INTRACELLULAR CALCIUM TRANSIENT IN FERTILIZING VOLTAGE-CLAMPED EGGS OF *Lytechinus pictus*

Holding potential (mV)	Latency ^a (sec)	Time to ^a peak (sec)	Ca _i at I _{sh} (μM)	Ca _i at peak (μM)	n
-20	36 ± 1.0	46 ± 4.3	0.8 ± 0.2	1.3 ± 0.2	3
-30	38 ± 3.6	46 ± 3.5	1.0 ± 0.4	1.6 ± 0.21	9

Note. Means and SEM are shown.

^a Latency is time from I_{on} to barely noticeable (20 nM) increase in Ca_i. Time to peak is time from barely noticeable Ca_i increase to peak of Ca_i transient.

I_{sh} is measured in control eggs, a statistically significant difference between the inward current in BAPTA-injected eggs and in controls is apparent (43 sec after I_{on} in these experiments). Inward current in BAPTA injected eggs does not increase significantly between t_{ca} and t_{sh}, while in control eggs inward current is increasing: midway between t_{ca} and t_{sh} it is 550 ± 50 pA (n = 5). This comparison suggests that the phase 1 current has a calcium-dependent component after the end of the latent period, when Ca_i begins to increase. I_m, the inward current maximum in BAPTA-injected eggs occurs, on average 10 sec before I_{peak}, the peak of the phase 2 current that, in control eggs, coincides with the Ca_i peak. I_m also precedes I_{peak} in *L. variegatus* (Table 3).

In Voltage-Clamped Eggs Injected with Neomycin Sperm Cause Inward Currents of Abrupt Onset and Rapid Cutoff

Figure 5 shows that eggs injected with neomycin (5–10 mM) and voltage clamped at -20 mV also show in-

ward currents after insemination. Inward currents were associated with single sperm which attached to the egg surface. They started with an abrupt I_{on} similar to that seen in EGTA-injected or normal eggs. However, in contrast to the currents in EGTA-injected or normal eggs (Lynn *et al.*, 1988), there was only a moderate increase in current to I_m; I_m was not much greater than I_{on}. In most cases the inward current returned to the preinsemination holding current level with an rapid decrease, or I_{off} (Fig. 5). This step-like on and off pattern of response occurred in both *L. pictus* and *L. variegatus* eggs, although again there was a slight difference in the magnitude of currents between the two species (see Table 2). In some cases, the sperm-induced inward currents showed a less distinct onset (14/61) or offset (4/61). As with EGTA-injected eggs, all of these neomycin-injected eggs failed to raise fertilization envelopes after insemination, allowing multiple sperm-egg interactions and inward current steps (I_{on}) to occur; a current induced by a second sperm could superimpose on an inward current caused by a previous sperm attachment.

Figure 6 shows that inward currents also occurred in neomycin-injected eggs (5–10 mM) when they were voltage clamped at -50 and -70 mV. Although the magnitude of currents is greater at more negative potentials (see Table 3), the basic pattern of response of these eggs to sperm is similar to that described for neomycin-injected eggs clamped at -20 mV. The majority of inward currents consist of an abrupt increase in holding current (I_{on}) followed by slow increase to I_m, then they were terminated by a rapid decrease (I_{off}) to the preinsemination current value (Fig. 6). As with currents at -20 mV these step-like inward currents could also be correlated with single sperm attaching to the egg surface. However, in contrast to sperm-induced currents at -20 mV, the currents in these eggs generally lasted less than 1 min. Figure 7A shows that the mean duration of the inward currents in neomycin-injected eggs was markedly voltage dependent, with 134-sec (mean duration) currents at -20 mV holding potentials and 19-sec (mean duration) currents at -70 mV. Figure 7B indicates that the shift in the mean duration of the currents

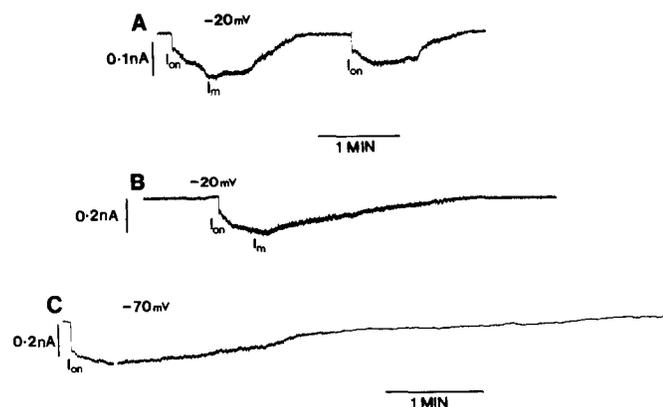


FIG. 3. Sperm-induced inward currents in EGTA-injected eggs. Each current has a distinctive I_{on} followed in some cases by a discernable I_m. Eggs are injected with 2–5 pl of a solution containing 100 mM EGTA (final concentration approx. 0.5–1 mM) under current clamp. They are then voltage clamped at -20 or -70 mV and inseminated with a low density of sperm. (A) Current record from a *L. pictus* egg clamped at -20 mV showing two successive sperm currents. (B) A record from a *L. variegatus* egg clamped at -20 mV. (C) A record from an *L. variegatus* egg clamped at -70 mV.

TABLE 3

MEMBRANE CURRENTS IN INSEMINATED VOLTAGE CLAMPED SEA URCHIN EGGS THAT HAD BEEN INJECTED PREVIOUSLY WITH EGTA OR NEOMYCIN

Experiment	Holding potential (mV)	I_{on} (nA)	I_m (nA)
<i>L. pictus</i> eggs			
EGTA-injected	-20	-0.030 ± 0.035 (12)	-0.124 ± 0.02 (9)
Neomycin-injected	-20	-0.035 ± 0.004 (5)	-0.05 ± 0.005 (9)
Neomycin-injected	-50	-0.148 ± 0.02 (15)	-0.210 ± 0.021 (19)
Neomycin-injected	-70	-0.273 ± 0.02 (16)	-0.420 ± 0.36 (21)
<i>L. variegatus</i> eggs			
EGTA-injected	-20	-0.106 ± 0.008 (17)	-0.332 ± 0.064 (8) ^b
EGTA-injected sperm enter	-70	-0.506 ± 0.036 (21)	-1.00 ± 0.054 (12) ^c
EGTA-injected sperm do not enter	-70	-0.385 ± 0.036 (9)	-0.565 ± 0.159 (5)
Neomycin-injected	-20	-0.087 ± 0.014 (12)	-0.175 ± 0.031 (8)
I_{sh}^a			
Noninjected controls	-20	-0.097 ± 0.008 (26)	-0.410 ± 0.023 (26) ^d
	-70	-0.450 ± 0.025 (7)	-0.910 ± 0.038 (7) ^d

Note. Means and SEM are given, with the sample number of sperm-induced currents in parentheses. The sizes of data samples are not matched in any one row because in some recordings either no clear I_{on} was seen despite an I_m and vice versa. Noninjected control data includes data published in Lynn *et al.*, 1988.

^a I_{sh} is the point of inflection that defines the boundary between phase 1 and phase 2 currents in control eggs (see Fig. 2).

^b Time from I_{on} to I_m : 22.2 ± 2.05 sec ($n = 8$).

^c Time from I_{on} to I_m : 22.1 ± 1.21 sec ($n = 12$).

^d Time from I_{on} to I_{sh} : 11.7 ± 0.3 sec ($n = 39$); to I_{peak} : 31.1 ± 0.9 sec ($n = 37$). All at 22–23°C.

at the different holding potentials is mainly due to the increased frequency of very brief duration (<15 sec) currents at more negative holding potentials and that the current durations are not symmetrically distributed about the mean, showing a long tail at long durations.

Short Duration Inward Currents in EGTA-Injected *L. variegatus* Eggs Voltage Clamped at -70 mV

The simple distinctions we have noted between the inward currents in EGTA- and neomycin-injected eggs were not all applicable in experiments on *L. variegatus* eggs injected with EGTA and held at a membrane potential of -70 mV during insemination. In fact, inward current responses in these eggs fell into two classes. The first class consists of the prolonged responses described earlier (see Fig. 3C and Table 3). The second class of response was observed in 3 of 10 eggs (9/30 current episodes). It consisted of an inward current with I_{on} and I_m comparable to the first class, but with a rapid cutoff (Table 3). Characteristically these responses were of short duration and resembled the sperm transient currents observed in *L. variegatus* eggs (Lynn *et al.*, 1988) or the episodic responses described for *L. pictus* eggs (David *et al.*, 1988) clamped at negative potentials.

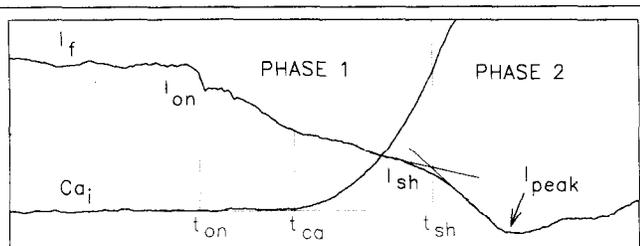
These responses also were quite similar, both in pattern and duration, to those described in neomycin-injected eggs clamped at -70 mV (see Fig. 6B, Table 3, and Fig. 7A). In these cases, no fertilization cone nor other evidence of sperm incorporation or of activation was detected and the sperm either remained attached to the external surface of the egg or floated away.

The Current-Voltage Relation of the Sperm-Induced Currents in EGTA-Injected Eggs Suggest That the Sperm-Associated Channels Inactivate

The current-voltage relation of the sperm-induced current in an EGTA-injected egg is shown in Fig. 8A. The reversal potential of the currents in seven *L. pictus* eggs was $-4 ± 1$ mV (mean and SEM). The sperm conductance declined gradually from a maximum at I_m and, when measured 30–60 sec after the return to preinsemination levels, the current voltage relation was indistinguishable from that of the unfertilized egg membrane (Fig. 8A). These observations, along with the data presented above, suggest that the sperm-induced conductance in EGTA-injected eggs slowly inactivates as the sperm is incorporated.

TABLE 4

MEMBRANE CURRENTS IN INSEMINATED VOLTAGE-CLAMPED SEA URCHIN EGGS (*L. pictus*) THAT HAD BEEN INJECTED PREVIOUSLY WITH 5 mM BAPTA, COMPARED TO CONTROLS



Fertilization control (s)	t_{on}	t_{ca}	t_{sh}	t_{peak}
	0	26±4	43±3	55±6
n=5 (pA)	$I_{t_{on}}$	$I_{t_{ca}}$	$I_{t_{sh}}$	$I_{t_{peak}}$
	84±18*	430±60%	650±60#	910±60=
5 mM BAPTA (pA)	$I_{t_{on}}$	$I_{t_{ca}}$	$I_{t_{sh}}$	I_m
	69±5*	380±70%	380±60#	470±60=
n=5 (s)	t_{on}	—	—	t_m
	0	—	—	44±7

Note. The diagram indicates the relation between membrane current and Ca_i in inseminated eggs. Currents are compared at times t_{on} , t_{ca} (when Ca_i is first detected to increase) and t_{sh} . t_m (BAPTA eggs) and t_{peak} (control eggs) are also shown; note that t_m precedes t_{peak} . *, %, not significantly different (Student's t test; $P > 0.4$). #, =, significantly different ($P < 0.02$)

The Current-Voltage Relation of the Sperm-Induced Currents in Neomycin-Injected Eggs Suggest That a Transient Conductance Appears in the Egg Membrane and Disappears Abruptly

The current-voltage relation of a sperm-induced current in a neomycin-injected egg at various times is shown in Fig. 8B. As with EGTA-injected eggs, the current-voltage relation was slightly inwardly rectifying during the inward current and the reversal potential for the current in eight *L. pictus* eggs was -4 ± 1 mV. Fifteen seconds after the sperm-induced conductance returned to preinsemination levels, the current-voltage relation was again indistinguishable from that of the unfertilized egg. These observations, along with those already presented which showed that sperm did not incorporate into neomycin-injected eggs and indeed, on

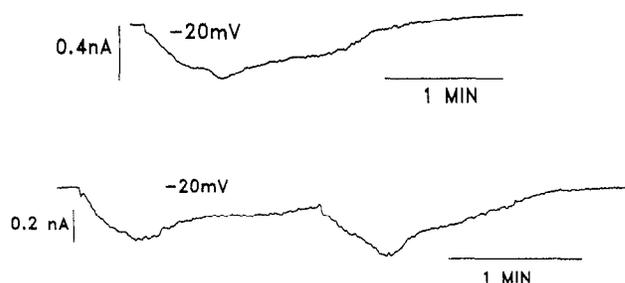


FIG. 4. Membrane currents in eggs microinjected with the calcium chelator BAPTA to a final concentration of 5 mM and voltage clamped at a holding potential of -20 mV (cf. Fig. 3). Two experiments are shown. In the upper trace, a single sperm has interacted with the egg. In the lower trace, two successive sperm-egg interactions occur. *L. pictus*; 16°C .

occasion, swam away, suggest that the sperm introduces only a transient conductance into the egg membrane in neomycin-injected eggs.

In parallel experiments in uninjected eggs the reversal potential of the slightly inwardly rectifying sperm-induced current measured during the first 5 sec after onset in *L. pictus* eggs was 2 ± 2 mV (mean and SEM, $n = 7$), suggesting that a similar cation conductance to that seen in EGTA- and neomycin-injected eggs is seen during the earliest stages of sperm-egg interaction.

DISCUSSION

The wave of fertilization envelope elevation in sea urchin eggs that is caused by the entering sperm can be prevented by microinjection of EGTA- or neomycin-containing solutions (Zucker and Steinhardt, 1978; Hamaguchi and Hiramoto, 1981; Swann and Whitaker, 1986). Here we report that sperm are still capable of causing inward currents under these conditions, when

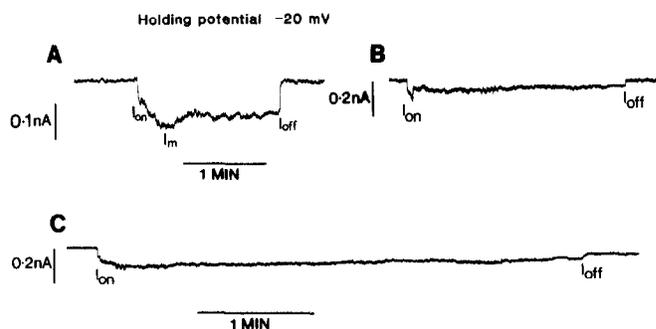


FIG. 5. Sperm-induced inward currents in neomycin-injected eggs voltage clamped at -20 mV. Eggs are injected with neomycin solution to give cytoplasmic concentrations of 5–10 mM and then inseminated under voltage clamp at -20 mV. (A) A current record from an *L. pictus* egg showing a rapid onset, I_{on} , and rapid cutoff, I_{off} . (B) A similar record from an *L. variegatus* egg. (C) A record from another *L. variegatus* egg with a rapid onset, but a more delayed and slower cutoff.

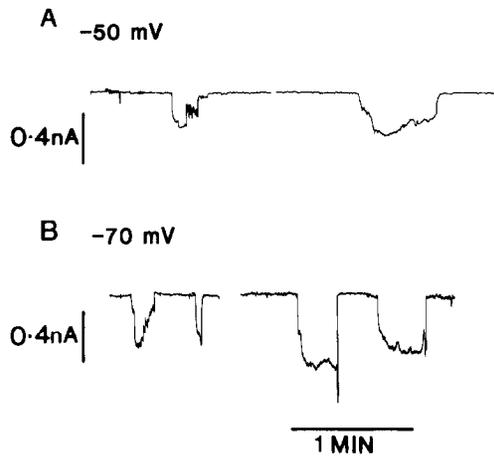


FIG. 6. Sperm-induced inward currents in neomycin-injected eggs of *L. pictus* at potentials more negative than -20 mV. Eggs are injected with 5–10 mM neomycin and clamped at different holding potentials before insemination. (A) Inward currents seen in a single egg clamped at -50 mV. (B) Inward currents from another egg clamped at -70 mV.

the fertilization calcium wave is prevented. Our results were qualitatively similar in both *L. pictus* and *L. variegatus* eggs. The sperm-induced currents in activation-inhibited eggs resemble the early phase (phase 1) of inward current in uninjected eggs (Lynn *et al.*, 1988; David *et al.*, 1988). In both EGTA- and neomycin-injected eggs a characteristic step-like increase in current, or I_{on} , is seen; no exocytosis or phase 2 inward current occurs. It appears that both EGTA and neomycin block events occurring during, or at the end of, the latent period at fertilization.

EGTA's effects can be fairly straightforwardly ascribed to its ability to chelate any calcium ions released in the egg. It is not as evident why neomycin blocks fertilization. Its effects must be reasonably specific, since neomycin-injected eggs have a resting membrane potential and membrane resistance comparable to controls. They also maintain their low, resting cytoplasmic calcium concentration and have an intact and functional internal calcium store (Swann and Whitaker, 1986), implying that they are metabolically competent. Neomycin may act by inhibiting the hydrolysis of polyphosphoinositides (Schacht, 1976; Whitaker and Aitchison, 1985), but it may also have an effect on cell membranes due to its ability as a polycation to alter membrane surface charge (McLaughlin and Whitaker, 1987). The ability of both EGTA and neomycin to arrest egg activation and the fertilization Ca_i transient allows us to examine and dissociate some of the earliest events in sea urchin fertilization.

Sperm-Induced Currents in EGTA-Injected Eggs

Characteristic sperm-induced currents in EGTA-injected eggs were associated with sperm incorporation and the formation of a large fertilization cone. Actin-rich fertilization cones have also been reported in EGTA-injected eggs of *Clypeaster japonicus* using fluorescently labeled actin (Hamaguchi and Mabuchi, 1988). The magnitude and time course of the rising phase of the current indicate that it closely resembles the form of the phase 1 fertilization current during the latent pe-

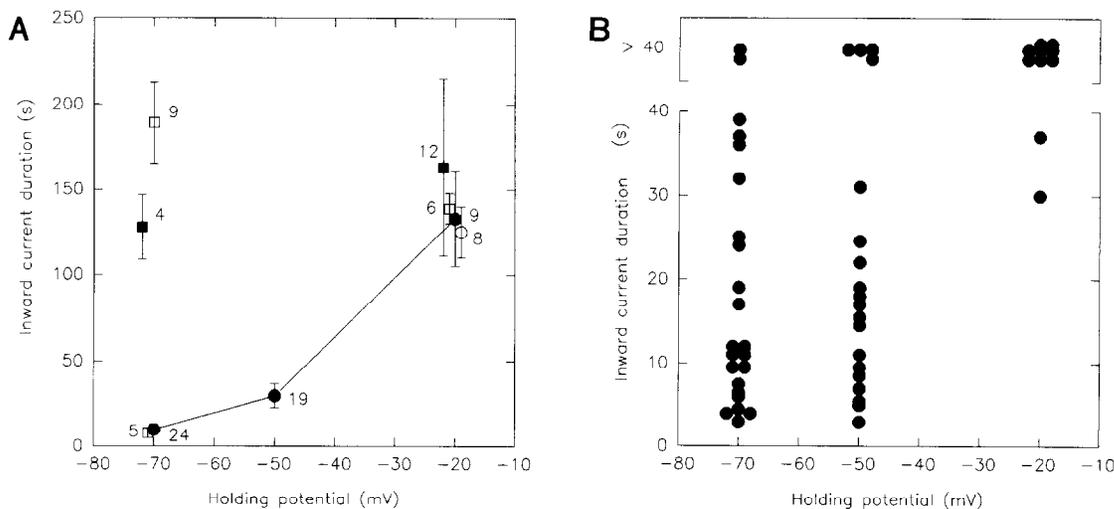


FIG. 7. (A) The duration of sperm-induced inward currents in voltage-clamped sea urchin eggs injected with either EGTA or neomycin. Eggs were voltage clamped at holding potentials of -20 , -50 , and -70 mV. Circles represent neomycin-injected eggs and squares represent EGTA-injected eggs. Data are shown separately for *L. pictus* eggs (filled symbols) and *L. variegatus* eggs (open symbols). The data represent the mean duration and standard errors of the means of individual currents that were judged to have been caused by single sperm-egg interactions. The numbers beside each symbol indicate the number of currents measured. (B) The distribution of duration of sperm-induced inward currents in neomycin-injected eggs clamped at -20 , -50 , and -70 mV. The distribution is skewed, with a tail at long durations.

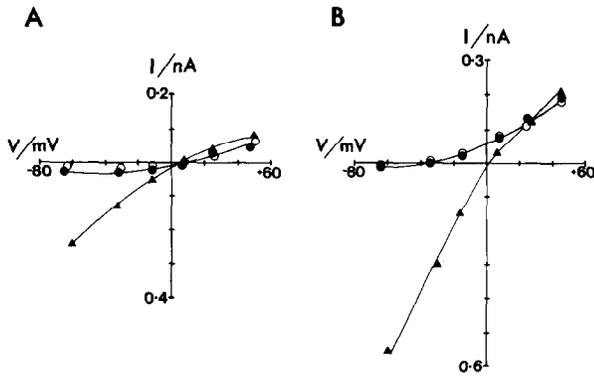


FIG. 8. The current-voltage relations of sperm-induced inward currents in *L. pictus* eggs. (A) The current-voltage relation of an EGTA-injected egg observed >5 min before (\bullet), during (1 min after I_{on} \blacktriangle), and 30 sec after (\circ) a sperm-induced inward current. (B) The current-voltage relation of a neomycin-injected egg plotted 20 sec before (\bullet), during (20 sec after I_{on} \blacktriangle), and 15 sec after (\circ) a sperm-induced inward current.

riod, before Ca_i increases (Lynn *et al.*, 1988 and this report); I_{sh} and the phase 2 current are absent, confirming the idea that the phase 2 current represents a calcium-activated cation conductance that is associated with the fertilization calcium wave (Lynn *et al.*, 1988; David *et al.*, 1988; McCulloh and Chambers, 1991). Calcium-activated cation conductances have also been identified at fertilization in hamster eggs (Miyazaki and Igusa, 1982), frog eggs (Kline, 1988), and eggs of the nemertean worm *Cerbratulus lacteus* (Kline *et al.*, 1986) using the calcium chelators EGTA and BAPTA.

The phase 1 fertilization current is the earliest detectable event at fertilization in sea urchin eggs and embodies the earliest phase of sperm-egg interaction. The phase 1 current is defined as the inward current that flows prior to the inflection (I_{sh}) that marks the beginning of the phase 2 fertilization current (Lynn *et al.*, 1988). The results that we present here indicate that this definition of the phase 1 current does not correspond exactly to the underlying activation events at fertilization. Thus, in *L. pictus* eggs, I_{sh} is observed to occur 10–15 sec after the end of the latent period, where the latent period is defined as ending with a barely noticeable Ca_i increase (Fig. 2 and Table 2). Ca_i at the time of I_{sh} is almost micromolar (Table 2). It appears that late phase 1 current has a large calcium-dependent component (Table 4) that, functionally, more properly belongs with the phase 2 current. The early phase 1 current occurs in both control and EGTA-injected eggs without any detectable increase in Ca_i and is unaffected by microinjection of up to 5 mM of the calcium chelator BAPTA. By these criteria, the current represented by I_{on} and the subsequent 10–15 sec of early inward current during the latent period at fertilization is a calcium-independent current.

For comparison, 5 mM BAPTA largely suppresses the calcium-activated potassium conductance in adrenal chromaffin cells (Marty and Neher, 1985), while 1 mM EGTA is sufficient to block the calcium-dependent potassium conductance in hamster eggs (Karl Swann, unpublished observations) and >0.7 mM BAPTA is sufficient to block the calcium-dependent cation conductance in frog eggs (Kline, 1988). We cannot rule out completely the possibility that a very large but local increase in Ca_i at the point of sperm attachment may be responsible for some or all of the early phase 1 current, since it is possible to argue that such an increase might be resistant to calcium chelators and undetectable using our methods. However, the precedents cited above lead us to think that this is unlikely.

If an increase in Ca_i is not a likely explanation for the abrupt appearance of the phase 1 current, then what other mechanisms are available? Membrane capacitance measurements in sea urchin eggs have suggested that sperm-egg membrane fusion is coincident with I_{on} of the phase 1 current (McCulloh and Chambers, 1992): the presence of sperm-induced currents in EGTA-injected eggs is clearly consistent with this suggestion, given that sperm fuse with and penetrate the eggs (Table 1; Kline, 1988). The capacitance measurements argue that the early, calcium-independent part of the phase 1 current results from the insertion into the egg membrane of channels present in the sperm membrane (McCulloh and Chambers, 1992). The sperm-induced current in EGTA-injected eggs most likely has the same basis. The slow decrease in the sperm-induced currents in EGTA-injected eggs may reflect the closing of these sperm channels or their physical removal from the fertilization cone, since after the decay of the inward current, the current-voltage relation of the egg membrane resembles that of the unfertilized egg. We noted that the duration of inward currents in EGTA-injected eggs into which sperm incorporated was not voltage dependent, indicating that the time for which the sperm-gated channels remain open after sperm-egg fusion is membrane potential insensitive.

Transient Sperm-Induced Currents in Control and EGTA-Injected Eggs

There is clear evidence that there is a high probability of a transient interaction between sea urchin sperm and egg when the egg membrane is held at the resting potential of -70 mV using a voltage clamp (Lynn *et al.*, 1988; David *et al.*, 1988; Whitaker and Crossley, 1990; Chambers and McCulloh, 1990). The transient interaction manifests itself as a phase 1 current, followed by an I_{off} , as in neomycin-injected eggs. Sperm fail to incorporate, but the transient interaction can cause egg activa-

tion (the phase 2 current and fertilization envelope elevation), often tens of seconds after I_{off} . The capacitance measurements show that sperm and egg are connected by an electrically conducting pore for the entire duration of each transient interaction [I_{on} to I_{off}] (McCulloh and Chambers, 1992).

We also observed transient sperm-egg interactions with sharp I_{off} and failure of sperm incorporation in EGTA-injected eggs held at -70 mV, although the probability of these unsuccessful interactions was strikingly smaller than in control eggs held at the same membrane potential. This observation supports the idea that a calcium influx antagonizes sperm incorporation (Chambers and McCulloh, 1990; McCulloh *et al.*, 1990); experiments that took it as a starting point will be reported elsewhere.

Sperm-Induced Currents in Neomycin-Injected Eggs

In neomycin-injected eggs, the sperm-induced currents were not associated with incorporation of the sperm or fertilization cone formation. The presence of inward currents in neomycin-injected eggs in the absence of sperm entry at first sight argues against sperm-egg fusion as the source of the phase 1 current: the presence of sperm-induced inward currents implies the formation of an aqueous, electrically conducting pore between egg and sperm (McCulloh and Chambers, 1992), while our Hoechst dye transfer assay confirmed our visual observations in voltage-clamped eggs and indicates that no cytoplasmic continuity is detectable between egg and sperm by this method. However, the two results can, in fact, be reconciled, if one accepts the possibility that sperm-egg fusion is, at least initially, a reversible process.

The most striking characteristic of the sperm-induced current in neomycin-injected eggs is its abrupt offset, I_{off} : a mirror image of the I_{on} that is characteristic of the phase 1 current in all experimental conditions. The timing of I_{off} in neomycin-injected eggs, that is, the duration of the sperm-induced current, is also markedly membrane potential dependent, being much reduced at negative holding potentials. There is an interesting parallel here with the observation we discuss above: that sperm fail to incorporate and eggs may fail to activate at negative holding potentials, despite the occurrence of phase 1 currents. The phase 1 currents during these unsuccessful sperm interactions are accompanied by changes in membrane capacitance that are consistent with cytoplasmic continuity between egg and sperm, just as during normal fertilization with sperm incorporation (McCulloh and Chambers, 1992).

There are strong similarities between the transient phase 1 currents in eggs held at more negative holding potentials and the transient currents in neomycin-in-

jected eggs. The observation that the initial sperm-egg interaction is labile at a membrane potential of -70 mV (Lynn *et al.*, 1988) suggests that the early, labile phase of sperm-egg interaction persists in neomycin-injected eggs, to such an extent that, after interacting with a neomycin-injected egg for a minute or more, a sperm can swim away. Perhaps the most interesting aspect of these labile interactions is the strong possibility that they represent the formation of a transient, reversible aqueous pore of the sort that has been described during fusion of secretory granules with the plasma membrane (Almers, 1990). The labile pore, if such it is in neomycin-injected eggs, does not survive chemical fixation (Hinkley *et al.*, 1986; Longo *et al.*, 1986) and, with a diameter of $0.06 \mu\text{m}$ estimated from its conductance (Whitaker *et al.*, 1989), is too small to permit significant transfer of the Hoechst dye used in the conventional fusion assay.

Sperm-Egg Interaction and the Fertilization Calcium Wave

The answer to the question of how the sperm initiates the fertilization calcium wave in sea urchin eggs lies in what occurs during the latent period. By extending the latent period indefinitely using neomycin and EGTA, we have shown that sperm-egg interaction can be assayed using the sperm-induced current. The calcium chelator favors sperm incorporation. Neomycin treatment reveals more clearly the initial, labile phase of sperm-egg interaction and its sensitivity to egg membrane potential. The egg membrane potential is an essential element of egg activation: a negative membrane potential is necessary to permit the earliest stage of sperm-egg interaction (Jaffe, 1976; Shen and Steinhart, 1984), while the depolarization triggered by the sperm current consolidates a transient interaction and in turn permits incorporation of the sperm into the egg (Chambers and McCulloh, 1990).

Our experiments do not permit us to decide unequivocally which of the two hypotheses of egg activation, receptor activation vs cytoplasmic messenger, best describes the events of fertilization. However, in our opinion, the membrane capacitance data (McCulloh and Chambers, 1992) and the abrupt appearance and disappearance of sperm-induced channels in the egg membrane during transient sperm-egg interactions favor the idea that egg activation results from the diffusion of a cytoplasmic messenger from egg to sperm through an aqueous pore (Dale *et al.*, 1985; Swann and Whitaker, 1990). If this hypothesis is to be sustained, it is important to determine the identity of the putative cytoplasmic messenger. A candidate activator has already been partially purified from hamster sperm (Swann, 1990).

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