

Fusion of Membranes during Fertilization

Increases of the Sea Urchin Egg's Membrane Capacitance and Membrane Conductance at the Site of Contact with the Sperm

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ABSTRACT The early events of fertilization that precede and cause activation of an egg have not been fully elucidated. The earliest electrophysiological change in the sea urchin egg is a sperm-evoked increase of the egg's membrane conductance. The resulting depolarization facilitates entry of the fertilizing sperm and precludes the entry of supernumerary sperm. The sequence of the increase in the egg's membrane conductance, gamete membrane fusion, egg activation, and sperm entry, including causal relationships between these events, are not known. This study reports the use of whole egg voltage clamp and loose patch clamp to monitor simultaneously changes of membrane conductance and capacitance at the site of sperm-egg contact. Measurements were made during sperm-egg interactions where sperm entry readily proceeded or was precluded by maintaining the egg's membrane potential either at large, negative values or at positive values. Whenever the sperm evoked an increase of the egg's membrane conductance, that increase initiated abruptly, was localized to the site of sperm attachment, and was accompanied by a simultaneous abrupt increase of the membrane capacitance. This increase of capacitance indicated the establishment of electrical continuity between gametes (possibly fusion of the gametes' plasma membranes). If sperm entry was blocked by large negative membrane potentials, the capacitance cut off rapidly and simultaneously with a decrease of the membrane conductance, indicating that electrical continuity between gametes was disrupted. When sperm entry was precluded by positive membrane potentials, neither conductance nor capacitance increased, indicating that sperm entry was halted before the fusion of membranes. A second, smooth increase of capacitance was associated with the exocytosis of cortical granules near the sperm in eggs that were activated. Electrical continuity between the gametes always preceded activation of the egg, but transient electrical continuity between the gametes alone was not always sufficient to induce activation.

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INTRODUCTION

Early during fertilization of sea urchin eggs, the fertilizing sperm attaches to the egg and then, after a brief (10–30 s) latent period (Allen and Griffin, 1958), the egg begins to activate. The early events that lead up to and cause activation have not been fully elucidated.

In the sea urchin egg, an increase of membrane conductance that occurs within seconds after sperm attachment is the earliest physiological event indicating intimate interaction between the sperm and the egg. The increase of conductance induced by the sperm depolarizes the egg from a resting value near -70 mV (Chambers and de Armendi, 1979; Lynn and Chambers, 1984; Lynn, McCulloh, and Chambers, 1988). Depolarization initiates an activation potential consisting of three distinct phases (Chambers and de Armendi, 1979). Phases 1 and 2 maintain the membrane potential more positive than $+10$ mV as the fertilization envelope elevates over the surface of the egg. Phase 3 returns the membrane potential to a value near the prefertilization resting membrane potential.

Neither the events that cause the early increases of membrane conductance nor the localization of the ion channels responsible for the increased conductance are clear. One possibility is that the sperm's membrane channels are introduced into the egg's membrane during fusion of the sperm's plasma membrane with that of the egg. It is unclear when the sperm plasma membrane is incorporated into the egg's plasma membrane by membrane fusion events. Also, it is not known whether the steps at which sperm entry is precluded by positive (Jaffe, 1976; Lynn et al., 1988) and/or negative (Lynn et al., 1988) membrane potentials precede or follow sperm–egg membrane fusion.

The purpose of the work described in this paper was threefold: first, to determine the location of the ion channels responsible for the currents and conductance increases during phase 1; second, to determine the time of sperm–egg plasma membrane fusion for sperm that enter the egg; and third, to determine if sperm–egg fusion precedes the steps at which sperm entry is blocked at positive and negative membrane potentials.

Our work involved the use of the loose patch clamp to monitor changes of membrane conductance and capacitance in a limited patch of membrane to which the fertilizing sperm attached and fused, and through which the sperm entered and activated the egg. The results indicate that (a) the channels responsible for increases of currents and conductance during phase 1 of the activation current are localized at or in the immediate vicinity of the site of sperm attachment; (b) electrical continuity between the gametes' cytoplasm is established at the time of I_{on} , the earliest physiological sign of intimate contact between sperm and egg; (c) at negative membrane potentials when sperm fail to enter the egg, electrical continuity is first established between the gametes at I_{on} , but is then disrupted; and (d) at positive membrane potentials that preclude both sperm entry and an electrophysiological response, fusion of the gametes does not occur. This is the first report of the use of capacitance techniques to study individual gamete–gamete membrane fusion.

Some of the results have been reported previously in abstract form (McCulloh and Chambers, 1986a, b).

Background

The following summarizes the responses in sea urchin eggs inseminated when the membrane potential is maintained constant by voltage clamp at values between -90 and $+30$ mV. This information facilitates an understanding of the objectives and results of the experiments presented in this paper. When the membrane potential of the egg is clamped at a value between -80 and $+17$ mV, three types of electrophysiological responses occur after the attachment of a sperm to the surface of the egg (Lynn et al., 1988; Chambers, 1989). Type I activation currents, which are accompanied by entry of the sperm (Lynn et al., 1988), occur almost exclusively at potentials between -20 and $+17$ mV. Type I activation currents also occur, in a decreasing proportion of the trials, throughout the range of membrane potentials between -20 and -80 mV. At membrane potentials increasingly more negative than -20 mV, type II sperm transient currents and type III modified activation currents make up an increasing proportion of the electrophysiological responses (Lynn et al., 1988). Both type II and III responses are associated with failure of the sperm to enter. At potentials more positive than $+17$ mV, neither electrophysiological responses nor sperm entry occur (Jaffe, 1976; Lynn et al., 1988). Brief descriptions of these types of electrophysiological responses follow.

Three phases are defined for the type I activation currents associated with sperm entry (Lynn et al., 1988). These three phases correspond to the three phases of the activation potential (Chambers and de Armendi, 1979; Lynn et al., 1988). Phase 1, of approximately the same duration as the latent period, is characterized by an abrupt onset of inward current, I_{on} , immediately followed by a slow increase of inward current for roughly 12 s to the level I_{sm} . Phase 2 begins as a more rapid increase of inward current from the level attained at I_{sm} and culminates with the attainment of the peak inward current, I_p , ~ 31 s after I_{on} . During phase 2, the fertilization envelope elevates as a wavefront that progresses over the surface of the egg and indicates that the egg is activated. During the first 2–3 s of phase 3, current declines precipitously to a level near that of I_{sm} . This is followed by a slow decrease and reversal of current over the subsequent 10–20 min for eggs clamped at membrane potentials more positive than the potassium ion equilibrium potential (~ -70 mV). The ion channels responsible for the second phase of the activation current open briefly in a wave that progresses over the surface of the egg from the site of sperm attachment to the opposite pole (McCulloh and Chambers, 1991). The location of the ion channels responsible for the earliest conductance increases during phase 1 is not known.

The currents observed when sperm fail to enter are different from those observed for sperm-egg interactions that result in sperm entry (Lynn and Chambers, 1984; McCulloh, Lynn, and Chambers, 1987; Lynn et al., 1988; Chambers, 1989). Failure of sperm entry is associated with two types of electrophysiological responses: type II sperm transient currents and type III modified activation currents (Lynn et al., 1988). For both types of responses, phase 1 begins with an abrupt onset of inward current, I_{on} , followed by a slow increase, the same as for type I responses. However, in marked contrast, phase 1 is then terminated by a rapid cutoff of the current. Type II sperm transient currents consist of this one phase in isolation with cutoff of the current to

the preinsemination value, the egg otherwise remaining in the unfertilized state. For type III modified activation currents, the cutoff of current at the end of phase 1 is to a value close to, but usually slightly more inward than, the preinsemination value. During phase 2, after a delay of 2–30 s, the inward current starts to increase, culminating in a maximum at I_p . Elevation of the fertilization envelope starts during this phase. Following I_p , the current decreases precipitously as phase 3 begins, again attaining a value close to the preinsemination level. For eggs clamped at potentials more positive than -70 mV, the current slowly reverses over a period of 10–30 min.

The failure of sperm to enter with type II and III responses is reliably predicted by the cutoff of current that terminates phase 1 (Lynn et al., 1988). The nature of the contact between sperm and egg leading to phase 1 in all three types of responses is not known. Fusion between sperm and egg plasma membranes is a prerequisite for sperm entry (Colwin and Colwin, 1967), but whether fusion is required for the conductance increase to occur is not known. It has been suggested that sperm–egg fusion occurs near the end of the latent period (Epel, Cross, and Epel, 1977; Whitaker and Steinhardt, 1985), at the time that the attached sperm's motility stops. However, electron microscopy has shown that cytoplasmic continuity between sperm and egg occurs earlier, by 5–8 s after I_{on} (Longo, Lynn, McCulloh, and Chambers, 1986). This is consistent with conclusions based on observations of the transfer of fluorescent dye from the egg to the sperm (Hinkley, Wright, and Lynn, 1986).

Sperm neither enter nor cause increases of membrane conductance in eggs maintained at membrane potentials more positive than $+17$ mV (Jaffe, 1976; McCulloh et al., 1987; Lynn et al., 1988). In eggs that are not voltage clamped, the rapid depolarization to a positive membrane potential induced by I_{on} , and the maintenance of the positive membrane potential throughout phases 1 and 2 serve as rapid blocks to the entry of supernumerary sperm (Jaffe, 1976; Lynn et al., 1988; McCulloh, 1989). A permanent block to polyspermy is provided by elevation of the sperm-impenetrable fertilization envelope. The fertilization envelope starts to elevate as phase 2 begins, but does not completely surround the egg until early during phase 3, as repolarization of the egg begins.

The differences between the membrane events accompanying failure of sperm entry at positive, contrasted with negative, membrane potentials suggest that sperm entry is precluded by voltage-dependent blockades at two distinct stages, before (for positive membrane potentials) or after (for negative membrane potentials) the occurrence of an electrophysiological response (Lynn et al., 1988). However, in neither case is it clear whether sperm entry is blocked before or after the fusion of sperm and egg membranes.

Clarification of Terms

In this paper, we deal with the issue of sperm–egg fusion. It must be made clear exactly what is meant by terms that might be considered synonymous with sperm–egg fusion. Sperm–egg fusion for sea urchin gametes involves the fusion of the plasma membrane of the tip of the acrosomal process of the sperm with the plasma membrane of the egg (Colwin and Colwin, 1967; Longo et al., 1986). When this membrane fusion process is complete, a state is achieved in which the plasma membranes surrounding the sperm and the egg are continuous (membrane continu-

ity). Electron micrographs would reveal continuous membranes as a single electron dense bilayer surrounding both gametes. The fusion of membranes results in the formation of a cytoplasmic bridge between the sperm and the egg (cytoplasmic continuity), such that solutes, including electrolytes and macromolecular constituents of the gametes' cytoplasms, are free to diffuse from one gamete to the other. Subsequent to sperm-egg fusion, the cytoplasmic bridge that provides cytoplasmic continuity becomes large enough to permit the sperm's organelles such as the nucleus, mitochondrion, and microtubule organizing center (centriole) to migrate into the cytoplasm of the egg. The fertilization process is completed at syngamy by intermixing of the genetic material of the sperm and egg.

Cytoplasmic continuity can be detected in living preparations by measurements of capacitance. An increase of capacitance of the patch indicates the establishment of an electrical connection (electrical continuity) between the cytoplasms of the sperm and the egg with millisecond time resolution. Electrical continuity occurs when continuity is established between the aqueous phases, including dissolved electrolytes, of the apposed gametes. This can be achieved not only by fusion of the gametes' membranes but also by the formation of gap junctions or pores that span the apposed membranes and which are permeable to small solutes (Bennett and Goodenough, 1978; Loewenstein, 1981) but not to macromolecules. Therefore, establishment of electrical continuity that we measure is not, by itself, unequivocal evidence that sperm-egg fusion has occurred. We have been careful in our use of these terms so as not to overstate our observations.

GLOSSARY

α	phase angle for current phasor for G
Θ	phase angle for current phasor for G_s
τ	time required for exponential decline from a maximum to $1/e$ of the maximum
ω	angular frequency
C	capacitance of the membrane patch
ΔC	change of the capacitance of the membrane patch
Δ imaginary	change of the amplitude of the imaginary component of admittance
C_p	pipette capacitance
G	conductance resulting from seal conductance and G_m in parallel
G_m	conductance of the membrane patch
G_s	series conductance ($1/R_{pip}$)
I	current
I_m	maximum amplitude attained during a type II or phase 1 of a type III response
I_{on}	amplitude of current at the abrupt onset of a sperm-induced electrophysiological response
I_p	amplitude of current at the peak of phase 2
I_{pip}	pipette current
I_{sm}	amplitude of current just before onset of phase 2
imaginary	imaginary part of admittance
j	square root of -1

membrane patch	the small region of the egg's plasma membrane that is circumscribed by the tip of the loose patch clamp pipette
phase 1	period during the egg's electrophysiological response induced by sperm, beginning with the abrupt inward current (I_{on}) and ending either with the onset of phase 2 (type I activation currents) or with cutoff of the current to a value near the preresponse level (type II and III responses)
phase 2	period during type I or III electrophysiological responses beginning with a second inward current increase (following phase 1 inward current) and ending with attainment of a maximum inward current (I_p) just before a precipitous decline of inward current toward the preresponse level
R_{pip}	resistance between the interior of the loose patch clamp pipette and the bath measured before pushing the pipette's tip against the surface of the egg
R_{seal}	resistance of the seal between the pipette and the egg measured as described previously (McCulloh and Chambers, 1991)
real	real part of admittance
type I activation current	the sperm-evoked electrophysiological response associated with sperm entry, consisting of three uninterrupted phases of inward current (phases 1, 2, and 3)
type II sperm transient current	a sperm-evoked electrophysiological response associated with failure of the sperm to enter the egg, consisting of one phase of inward current that terminates rapidly
type III modified activation current	a sperm-evoked electrophysiological response associated with failure of the sperm to enter the egg, consisting of three phases of inward current (phases 1, 2, and 3) for which phase 1 rapidly cuts off before the completion of phases 2 and 3
V_m	membrane potential of the whole egg
V_{pip}	difference of potential between the interior of the pipette and the bath
whole egg/cell currents	currents recorded with switched voltage clamp, including any currents that pass through the entire plasma membrane of the egg including the membrane patch

METHODS

Solutions

Seawater was collected from the Gulf Stream approximately five miles offshore from Miami, FL. Before use in experiments, it was passed through a filter (RA 1.2 μm ; Millipore Corp., Bedford, MA) and supplemented with 10 mM Tris(hydroxymethyl) methylaminopropanesulfonic acid (TAPS; Sigma Chemical Co., St. Louis, MO), and the pH was adjusted to 8.3.

Gametes

Sea urchins, *Lytechinus variegatus*, were collected from the waters near Miami, FL and were maintained in seawater aquaria for no more than several weeks before use as gamete donors. Gametes were collected, stored, and prepared for experiments as described previously (Lynn et al., 1988; McCulloh, 1989; McCulloh and Chambers, 1991). Only eggs and sperm that resulted in >95% fertilization envelope elevation and cleavage during test inseminations (McCulloh and Chambers, 1991) were used for the experiments in this paper.

Microscopy

Several hundred eggs were pipetted into a 25-mm Falcon Petri dish cover (Falcon Plastics, Cockeysville, MD) containing ~2 ml of seawater. The dish was mounted on the stage of an inverted microscope (Invertoscope; Zeiss, Oberkochen, Germany) and the eggs were viewed with a 40× long working distance planachromat objective lens. Under these conditions, sperm that attached to the surface of the egg were readily visible, especially if they attached to the surface of the egg in the plane of focus at which the egg had its greatest diameter. However, when sperm were added to the inside of the pipette (see below), observation of sperm attachment was much more difficult because the distortions caused by the curvature and refractive properties of the pipette made it difficult to distinguish attachment of the sperm to the egg from adherence to the inside surface of the pipette.

Electrophysiology

Loose patch clamp. The loose patch clamp technique (Neher and Lux, 1969; Fishman, 1975; Stühmer, Roberts, and Almers, 1983; Whitaker and Steinhardt, 1983; McCulloh and Chambers, 1991) was used to monitor currents, conductance, and capacitance of a localized membrane patch circumscribed by a patch clamp pipette pushed against the surface of the egg. Recording the capacitance of only the circumscribed membrane patch, in theory, makes detection of the sperm's capacitance less difficult because the surface area and capacitance of the membrane patch more nearly approximate the surface area and capacitance of the sperm membrane. It is advantageous to use a recording instrument with extended bandwidth for measurement of capacitance because the capacitive currents increase with frequency. Therefore an additional benefit of the loose patch clamp is that its bandwidth extends to ~5 kHz.

Loose patch clamp pipettes. Patch pipettes were pulled from disposable micropipettes (Boralex, 20-50-100 μl; Rochester Scientific Co., Rochester, NY) using a three-stage pull that created an abrupt taper just at the tip of the pipette. Heat polishing smoothed the broken edge of glass at the tip and decreased the inside diameter to ~10 μm so that the cross-sectional area of the orifice was ~7.9 × 10⁻⁷ cm². The steep taper was desired so that sperm introduced inside the pipette could gain access to the membrane patch surrounded by the tip, and so that the majority of the pipette's electrical resistance would be localized to the constriction at its tip. After heat polishing, the total length of the pipette was adjusted so that the polyethylene tube to be inserted inside the pipette would extend to within 200 μm of the tip. This was accomplished by breaking off the butt end of the pipette and heat polishing it to avoid damaging the delicate polyethylene tube with the sharp edges of an unpolished glass pipette.

Patch pipettes were coated with BSA inside and with polylysine outside the pipette. The inside of the pipette was coated by injecting a small drop of seawater that contained 0.3–3 mg/ml of BSA (Fraction V, crystallized; Sigma Chemical Co.) into the pipette near the beginning of the taper. This drop moved by capillary action to the tip of the pipette. As soon as possible after the drop reached the tip of the pipette, a 5-cm³ syringe was used to withdraw the BSA solution back toward the butt end of the pipette until it was removed. Suction was continued, maintaining the air flow into the tip of the pipette for several seconds in an attempt

to partially air dry the BSA near the tip of the interior of the pipette. The BSA coating was applied to prevent sperm from adhering to the walls of the pipette in the region near the tip.

The external surface of the pipette was coated with polylysine by dipping the tip of the pipette into a solution of 0.1% polylysine (70,000–200,000 mol wt; Sigma Chemical Co.) in water for ~30 s. Flow of polylysine solution into the tip of the pipette was avoided by forcing air from the inside of the pipette through the tip before, throughout, and after dipping. Air was forced through the pipette by pressure applied from the syringe attached to the butt end of the pipette. A continuous stream of small bubbles emanating from the tip of the pipette in the solution confirmed that the air flow was sufficient to avoid entry of polylysine.

After coating, the pipette was filled with seawater (from the tip to about half-way up the shaft) and mounted in the loose patch clamp's microelectrode holder. The holder had two Ag/AgCl wires (McCulloh and Chambers, 1991) and a small diameter polyethylene tube that contained a bolus of dry sperm surrounded by a gap of immersion oil on each end of the dry sperm bolus. The wires and tube were inserted through the large diameter end of the pipette into the interior of the pipette such that the wires made contact with the seawater. The polyethylene tube, which was longer than the wires, was carefully positioned within 200 μm of the tip of the pipette.

Placement of the loose patch clamp pipette and establishment of the loose seal. Suction was applied to the interior of the pipette to help maintain the seal between the pipette and the surface of the egg. Suction was controlled and maintained by a water-filled, U-tube manometer constructed of two vertical glass tubes joined at the bottom by plastic tubing. The inside diameter of the glass tubing was ~3 mm. One end of the manometer was connected via polyethylene tubing to the 90° sidearm of the pipette holder to apply suction to the interior of the pipette. Suction sufficient to maintain the seal without causing an omega-shaped bleb of egg to be drawn into the pipette was applied by creating a difference of 2–3 mm between the levels of water in the two glass tubes.

The potential of the bath was maintained at 0 mV by a virtual ground device that made contact with the seawater in the Petri dish containing the eggs via two Ag/AgCl wires (one to sense the potential and the other to pass current). This arrangement avoided significant charging of the Ag/AgCl-coated, voltage-sensing junctions during the passage of large currents.

Upon placing the pipette into the seawater within the Petri dish, small pulses were applied to the command voltage of the loose patch clamp (operational amplifier with a 10-M Ω feedback resistor) to determine the resistance of the pipette (R_{pip}). R_{pip} averaged 0.18 ± 0.021 M Ω ($n = 24$) for the experiments in which capacitance measurements were made. After determining R_{pip} , the pipette was pushed against the surface of the egg and suction (–2 to –3 mm of H₂O pressure) was applied to the interior of the pipette. The pipette was pushed forward to improve the seal, indenting the surface of the egg (Fig. 1). The indentation was less extreme in these experiments than it was previously (McCulloh and Chambers, 1991), but the tip of the pipette was often pushed to a position near the center of the egg. The resistance (R_{seal}) of the seal between the pipette and the surface of the egg was determined by applying voltage steps to the command potential of the loose patch clamp, dividing the amplitude of the voltage steps by the amplitude of the resulting current steps and subtracting R_{pip} . R_{seal} averaged 1.7 ± 0.14 M Ω ($n = 23$) and was $6.6 \pm 0.56 \times$ the value of R_{pip} ($n = 23$).

Due to the presence of microvilli, the surface area of the membrane is expected to be roughly twice the surface area (Schroeder, 1979) of a circle with the same diameter as the inside of the pipette tip, or ~157 μm^2 . The capacitance of the membrane patch estimated by multiplying surface area by 1 $\mu\text{F}/\text{cm}^2$ (Cole, 1972) is 1.57 pF. For reference, a ballpark estimate of the conductance for the membrane patch (~4 pS) can be made by multiplying the whole egg conductance (~2 nS) by the fraction (~0.002) of the egg surface in the membrane patch.

Voltage clamp of the whole egg. The membrane potential for the whole egg was maintained constant using a single microelectrode, switched voltage clamp (Dagan Corp., Minneapolis, MN; Wilson and Goldner, 1975) essentially as described previously (Lynn and Chambers, 1984; McCulloh et al., 1987; Lynn et al., 1988; McCulloh, 1989; McCulloh and Chambers, 1991). Microelectrodes were pulled using thin-walled glass (#30-30-0; Frederick Haer, Brunswick, ME). The outer surface of each microelectrode was painted with one coat of conductive silver paint extending ~ 1.5 cm along the shaft beginning ~ 200 μm from the tip. The conductive paint near the tip was insulated (from the seawater in the Petri dish) by painting over it to within 20–100 μm of the tip with polystyrene paint (Q-dope; GC Electronics, Rockford, IL). Upon filling with 0.5 M K_2SO_4 , 10 mM NaCl, and 0.1 mM Kcitrate, the resistances were ~ 15 –35 M Ω , and with compensation for microelectrode capacitance, microelectrode potential changes settled in < 70 μs . The silver paint was driven at the potential of a headstage output ($\times 1$) and thus served as a shield surrounding the microelectrode.

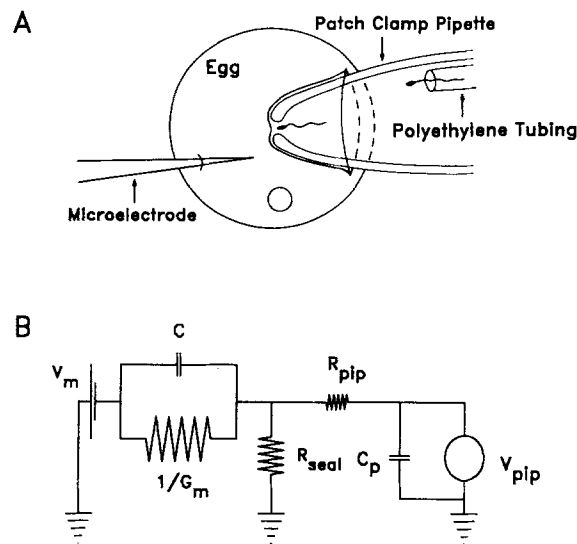


FIGURE 1. Diagram of recording configuration. (A) A loose patch clamp pipette was pressed against the surface of the egg. The pipette was advanced until it indented the egg's surface, thereby electrically isolating a patch of the egg's plasma membrane circumscribed by the tip of the pipette. A microelectrode was inserted into the cytoplasm of the egg to control the membrane potential of the egg. Sperm were introduced into the interior of the loose patch clamp pipette, assuring that any sperm-egg membrane interaction occurred with the

egg's membrane patch. (B) The equivalent electrical circuit for analysis of the admittance of the membrane patch. The circuit elements are defined in the Glossary.

Once a loose patch clamp pipette had been successfully pushed against the surface of an egg and a sufficient seal resistance had been attained, the whole egg voltage clamp's microelectrode was inserted into the egg as described previously (McCulloh, 1989; McCulloh and Chambers, 1991).

Once the membrane potential remained constant for > 2 –3 min in response to the applied current, the applied current was turned off, the resting membrane potential was noted, the command potential was set to the egg's resting membrane potential, and the voltage clamp was switched on. The switching frequency for the clamp was typically 1.5 kHz and the gain was raised as high as we could attain without fear of oscillation of the clamp.

Measurement of changes of the local membrane capacitance. Capacitance of the membrane patch was monitored by applying a sine wave (1 kHz, 20 mV peak to peak, centered at the bath potential) to the command potential for the loose patch clamp. (The membrane potential of the egg was maintained constant by the whole egg voltage clamp throughout this procedure.)

The use of a 1-kHz frequency greatly facilitated detection of changes in the capacitive currents that we did not detect above the noise using a 100-Hz sine wave. The switching frequency (~ 1.5 kHz) for the single-microelectrode, switched voltage clamp was not sufficient to control the whole egg at this high a frequency and hence the loose patch clamp that is capable of controlling the potential at up to 5 kHz was chosen as the recipient of the 1-kHz sine wave command voltage. The time constant of the egg (typically > 100 ms) is much slower than the switching frequency for the single-microelectrode, switched voltage clamp. Therefore, the whole egg's potential varies negligibly in response to the patch clamp's sine wave.

The majority of the currents in response to changes of command potential for the loose patch clamp flow through the seal resistance (McCulloh and Chambers, 1991) and the membrane and pipette capacitances (several picofarads). Compensation for these currents was applied directly, without feedback, to the loose patch clamp pipette, thereby avoiding saturation of the loose patch clamp's headstage amplifier and permitting more sensitive detection for changes of the properties of the membrane patch.

The sinusoidal currents recorded with the loose patch clamp were analyzed with the aid of a lock-in amplifier (model 840 Autoloc; Keithley Instruments, Inc., Cleveland, OH) using the loose patch clamp's command potential as a reference signal (Neher and Marty, 1982; Breckenridge and Almers, 1987; Lindau and Neher, 1988; Kado, 1989). The root mean square (rms) amplitudes of the currents leading the reference signal by 80 – 100° (the imaginary component of current) were filtered and displayed on a chart recorder (model 2200S; Gould Inc., Cleveland, OH).

Determination of the appropriate phase angle for detection of membrane capacitance increases.

The use of phase-sensitive detection to monitor membrane capacitance has been discussed previously (Neher and Marty, 1982; Fernandez, Neher, and Gomperts, 1984; McCulloh, 1985; Lindau and Fernandez, 1986; Breckenridge and Almers, 1987; Joshi and Fernandez, 1988; Lindau and Neher, 1988). Some equations presented herein appear in the article by Joshi and Fernandez (1988) but are of relevance to this paper and are included. The circuit described by Joshi and Fernandez (1988) involves basically four circuit elements: the conductance of the patch clamp pipette, in series with the membrane, (G_s); the capacitance of the patch clamp pipette (C_p); the conductance of the membrane patch (G); and the capacitance of the membrane patch (C). In our application, the elements assume the same meanings except that in this paper the membrane conductance is in parallel with the large, seal conductance and hence G is dominated by the conductance of the seal (Fig. 1). The resultant solution for the admittance of the circuit can be separated into real and imaginary parts (Joshi and Fernandez, 1988):

$$\text{real} = \frac{G(1 + G/G_s) + \omega^2 C^2/G_s}{(1 + G/G_s)^2 + (\omega C/G_s)^2} \quad (1)$$

$$\text{imaginary} = j\omega \left[C_p + \frac{\omega C}{(1 + G/G_s)^2 + (\omega C/G_s)^2} \right] \quad (2)$$

The current phaser for each circuit element is directed at an angle that can be estimated from the partial derivative of the admittance with respect to the circuit element. The solutions define the phase angle for changes of G (α) and the phase angle for changes of G , (Θ) (Joshi and Fernandez, 1988):

$$\alpha = -2 \tan^{-1} \left(\frac{\omega C}{G + G_s} \right) \quad (3)$$

$$\Theta = 2 \left[\tan^{-1} \left(\frac{\omega C}{G} \right) - \tan^{-1} \left(\frac{\omega C}{G + G_s} \right) \right] \quad (4)$$

These angles represent the angles at which the admittance measurements are most sensitive to changes of that particular circuit element. Phase angles orthogonal (90° shifted) to these represent phase angles which are insensitive to changes of that circuit element. The current phaser for C is orthogonal to α (Joshi and Fernandez, 1988). Therefore, by substitution of the known values (presented above) into these equations, estimates for the phase angles are obtained. α is estimated to be -0.2° and Θ is estimated to be 181.5° . The phase angle for changes of C is orthogonal to α and hence is estimated to be at -89.8° . The phasers lie within 1.5° of the real and imaginary axes. Therefore, the most appropriate phase angle for monitoring changes of capacitance is very near 90° . Monitoring the admittance at 90° is also the phase angle that yields the admittance for the imaginary component.

The major reason for such careful consideration of phase angles by Joshi and Fernandez (1988) is that they were interested in accurately monitoring the magnitude of many step increases of C . However, the optimal phase angles change with any changes of the circuit elements, including several step increases of C . Our interest is primarily with the first increase of C and therefore the measured values of G_s and G are likely to be good estimates of the values at the time of the change of C . A major benefit of using the loose patch clamp is that the measured changes of membrane conductance have little effect on G , thus eliminating the problems caused by changes of this conductance.

Eq. 2 can be simplified further if G_s is much greater than either G or ωC , thereby reducing the imaginary component to:

$$\text{imaginary} = j\omega(C_p + C) \quad (5)$$

C_p remains constant throughout the experiment. Therefore, changes of the imaginary component would be directly attributable to changes of the membrane capacitance such that:

$$\Delta C = \Delta \text{imaginary} / j\omega \quad (6)$$

Capacitance values stated in this paper were calculated using Eq. 6. The initial value of G_s (5.6×10^{-6} S) is much greater than ωC (9.9×10^{-9} S), but is only 6.6 times the initial value of G . By substituting these values into Eq. 2, it is shown that the values of C stated in this paper underestimate the actual capacitance changes by $\sim 25\%$.

Measurement of changes of the local membrane conductance. The conductance of the membrane patch was monitored as described previously (McCulloh and Chambers, 1991) by monitoring current steps in the loose patch clamp pipette held at the bath potential while the potential of the whole egg was stepped, using the single-microelectrode, switched voltage clamp.

Storage of electrophysiological data. Membrane potential for the whole egg currents (measured using the single-microelectrode, switched voltage clamp) membrane patch currents (measured using the loose patch clamp) and capacitance (the output from the lock-in amplifier) were each recorded on separate channels of a four-channel FM instrumentation tape recorder (model 3964A; Hewlett-Packard Co., Palo Alto, CA) so that they could be played back at a later time. Data were collected by computer (IBM-XT) using analog to digital conversion (Labmaster; Tekmar Co., Cincinnati, OH) of the signals played back from the tape recorder. These digitized values are displayed in the figures of this paper.

Insemination

A polyethylene tube (PE200, Intramedic; Clay-Adams Co., Parsippany, NJ) was heated and drawn to form a tube of much smaller diameter (~ 30 – $50 \mu\text{m}$ i.d.). The narrow tubing was cut to form a small diameter tip. This tube to be introduced to the interior of the loose patch clamp

pipette was connected via its large diameter end to a 3-ft section of polyethylene tubing (PE200) that was in turn connected to a 500- μ l micrometer syringe (Hamilton Co., Reno, NV) controlled manually via a threaded, screwdriven plunger. The entire syringe and length of tubing was filled with distilled water. A drop of dry sperm was placed on a glass slide along with a drop of immersion oil. The narrow tip of the polyethylene tube was first dipped into the immersion oil and oil was drawn into the tube to serve as a barrier between the distilled water and the sperm. Then a small bolus of dry sperm (100–300 μ m long) was drawn into the tube followed by a small plug of immersion oil to separate the dry sperm from the seawater in the interior of the loose patch clamp pipette. The loose patch clamp pipette was slipped over the polyethylene tubing such that the tip of the tubing was inside the loose patch clamp pipette within 200 μ m from its tip.

When the loose patch clamp pipette and the whole egg voltage clamp's microelectrode were in place and the egg's membrane potential was clamped at the desired potential, sperm were introduced into the interior of the pipette (Uehara and Katou, 1972) by forcing a small portion of the sperm bolus out of the polyethylene tubing. Attempts were made to stir the dry sperm by repeatedly drawing sperm back into the polyethylene tube and reexpelling them. Such increases and decreases of the volume of fluid in the pipette affected the pipette capacitance slightly, but readjustment of capacitance compensation was accomplished in the several minutes before sperm–egg contact (see Results).

In several cases where sperm could not be found in the polyethylene tube at the time when insemination was desirable, sperm were alternatively diluted in seawater (1 μ l dry sperm/40 ml seawater) and 100- μ l aliquots of this dilution were added to the 2-ml bath surrounding the egg.

Statistics

Values in this paper, unless explicitly stated, are expressed as the mean \pm standard error, with the number of observations, n , indicated in parentheses. Comparisons between means were made using analysis of variance (Sokal and Rohlf, 1973) unless stated otherwise.

Contingency tests for independence of events were performed using likelihood ratios (G tests; Sokal and Rohlf, 1973).

RESULTS

Currents recorded using the single-microelectrode, switched voltage clamp monitored the currents passing through the entire membrane of the egg, including those through the membrane patch (whole cell or whole egg currents). Currents recorded using the loose patch clamp monitored the currents passing through the patch of membrane circumscribed by the tip of the patch pipette (membrane patch or patch currents). This patch of membrane surrounded and included the site of sperm attachment, unless otherwise stated. Amplitudes of currents flowing into the egg are reported as negative values regardless of the method of measurement.

General Features Observed When Insemination Was Attempted by Introducing Sperm Inside the Loose Patch Clamp Pipette

Insemination from within the patch pipette was attempted for 63 eggs. For 26 eggs, interactions between sperm within the pipette and the egg resulted in electrophysiological responses. Generally, we concluded that the egg had a nearly 100% chance of undergoing an electrophysiological response caused by a sperm within the pipette if sperm could be introduced into the pipette and subsequently swim. The observed electrophysiological responses were similar to those observed after application of

sperm to the bath. The time between introduction of dry sperm from the polyethylene tube into the seawater within the patch pipette and occurrence of an electrophysiological response averaged 9.8 ± 1.9 min in these 26 cases. A histogram of these waiting times is well approximated by an exponential distribution (Fig. 2), characteristic of a random, Poisson process.¹ A time constant of 9.88 min was obtained by fitting using a method that maximizes likelihood that the data came from this distribution. The similarity of the average value (9.8 min) and the fitted time constant (9.88 min) argues that the data are well described by one exponential distribution. The likelihood used for fitting was not significantly improved by adding a second exponential distribution; therefore, it is unjustified to describe the data with more than one time constant.

Among the 63 attempts to fertilize the egg by introducing sperm into the pipette, 37 attempts failed for various reasons. The most common reason was failure to find sperm within the polyethylene tubing or within the pipette when the pipette and microelectrode had been positioned. We did not attempt to determine where the

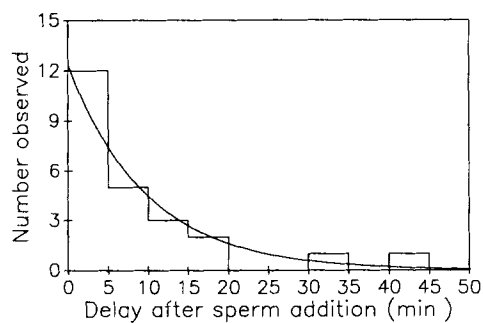


FIGURE 2. Histogram of the delays between sperm addition and occurrence of an electrophysiological response when sperm were introduced to the interior of the loose patch clamp pipette and an electrophysiological response occurred. Open bars represent the number of eggs observed. The smooth curve displays the best-fit exponential curve ($\tau = 9.88$ min) that approximates the data. The time constant defining the exponential

curve was obtained by a reiterative procedure that fitted the actual times with a single decaying exponential function using a maximum likelihood method.

sperm went. Occasionally sperm failed to swim once they were introduced to the seawater inside the loose patch clamp pipette. Sperm were added to the bath and successfully induced electrophysiological responses with the egg in 18 of the cases for which sperm could not be found inside the polyethylene tube. Experiments with the remaining 19 eggs were not continued for other reasons: poor insertion of the intracellular microelectrode, inability to voltage clamp the egg with the microelectrode, loss of the loose patch clamp pipette's seal resistance before insemination, formation of a large bleb within the patch clamp pipette, or frustration of the experimenter.

The single-microelectrode, switched voltage clamp was used to maintain the membrane potential for each egg at a constant value between -90 and $+45$ mV. Three ranges of membrane potential were investigated extensively: between -30 and 0 mV, between -90 and -60 mV, and between $+25$ and $+45$ mV. For the 12 eggs clamped at potentials between -30 and 0 mV, at an average membrane potential of

¹This distribution is used for statistical arguments in the section on failure of sperm entry at positive membrane potentials.

-14 ± 1.8 mV ($n = 12$), 12 eggs exhibited activation currents, 11 eggs developed asters within 10 min of the initial electrophysiological response, demonstrating that sperm entry had occurred, and 10 eggs subsequently divided to the two-cell stage or beyond (also indicating that the sperm had entered the egg). Several zygotes developed to the swimming blastula stage. In the group of 10 eggs held at potentials between -90 and -60 mV, averaging -82 ± 1.2 mV ($n = 10$), 4 eggs underwent type I activation currents, each developing a sperm aster within 10 min. Of these, three subsequently developed at least to the 2 cell stage. Sperm transient currents occurred in five eggs and a modified activation current in one egg, none of which developed sperm asters or cleaved. Excluded from the above are several eggs that underwent more than one response. For all seven eggs maintained between $+25$ and $+45$ mV, no responses were observed and sperm entry failed.

TABLE I
 Durations of Phases 1 and 2 during Type I Activation Currents Recorded for the
 Whole Egg Clamped at Membrane Potentials Between 0 and -30 mV
 (Mean = -14 ± 1.8 mV)

	Phase 1	Phase 2	Phases 1 + 2
Sperm inserted into pipette	19 ± 1.3^s ($n = 11$)	11 ± 1.2^s ($n = 10$)	30 ± 1.3^s ($n = 10$)
Sperm added to bath (Lynn et al., 1988)	12 ± 0.03^t ($n = 39$)	20 ± 0.8^t ($n = 35$)	31 ± 0.9^t ($n = 37$)

Values bearing the same superscript were not significantly different ($P > 0.05$). Values bearing the superscript * were significantly different from values bearing the superscript † ($P < 0.01$). Values bearing the superscript ‡ were significantly different from values bearing the superscript † ($P < 0.01$).

Current and Capacitance Measurements during Type I Responses Induced by Sperm That Entered the Egg from Within the Loose Patch Clamp Pipette

In this section, we describe the whole egg currents, patch currents, and changes of membrane capacitance for the patch that were recorded from eggs that underwent type I activation currents (Lynn et al., 1988) while held at potentials between -90 and 0 mV. The durations of phases (Table I) and the amplitudes of the currents (Table II) are described in detail for responses recorded from eggs maintained at potentials in the range -30 to 0 mV (mean = -14 ± 1.8 mV). Responses in eggs maintained at potentials between -90 and -60 mV (mean = -78 ± 2.5 mV) were essentially the same except that the amplitudes of the currents were larger at the more negative potentials (see Table III) due to the increase of driving force on the ions flowing through the membrane. Sperm entry was confirmed for eggs in both potential ranges by the presence of a sperm aster and subsequent cleavage of the egg (see above).

Whole egg currents in eggs clamped between -30 and 0 mV. The first sign of successful sperm-egg interaction during the type I activation current (Fig. 3A) was the occurrence of a small, abrupt inward current (I_{on}) -0.05 ± 0.008 nA ($n = 11$) in amplitude. The inward current continued to increase, slowly attaining a level (I_{sm}) of -0.26 ± 0.030 nA ($n = 11$) just before a more rapid increase of inward current beginning at 19 ± 1.3 s ($n = 11$) after I_{on} . This first period of abrupt onset and slow

TABLE II
*Amplitudes of Type I Activation Currents for the Whole Egg and the Membrane Patch
 Clamped at Membrane Potentials between -30 and 0 mV (Mean = -14 ± 1.8 mV)*

	I_{on}	I_{sm}	I_p
	nA	nA	nA
Whole egg, sperm in pipette	$-0.05 \pm 0.008^*$ ($n = 11$)	-0.26 ± 0.030^f ($n = 11$)	-1.6 ± 0.29^f ($n = 10$)
Membrane patch	-0.05 ± 0.011 ($n = 10$)	-0.28 ± 0.039 ($n = 10$)	n.a.
Membrane patch, corrected	$-0.06 \pm 0.013^*$ ($n = 9$)	-0.34 ± 0.039^f ($n = 9$)	n.a.
Whole egg, sperm added to bath (adapted from Lynn et al., 1988)	-0.10 ± 0.012^f ($n = 8$)	-0.27 ± 0.029^f ($n = 8$)	-1.3 ± 0.17^f ($n = 8$)

Values bearing the same superscript were not significantly different ($P > 0.05$). Values bearing * as the superscript were significantly different from the value bearing ^f as its superscript ($P < 0.025$). Comparisons were made using analysis of variance.

rise through the time that the current began to rise more rapidly is called phase 1. Phase 2 began at the end of phase 1 and was characterized by a rapid increase of current to a peak (I_p) of -1.6 ± 0.29 nA ($n = 10$) attained 30 ± 1.3 s ($n = 10$) after I_{on} . A rapid decrease of current after I_p terminated phase 2. The duration of phase 2 measured from the end of phase 1 to the time of occurrence of I_p averaged 11 ± 1.2 s ($n = 10$).

The type I activation currents just described (Fig. 3A) recorded from whole eggs inseminated by inserting sperm into the pipette exhibit the same pattern as described by Lynn et al. (1988), where sperm were added to the seawater surrounding the egg. The total duration of phases 1 + 2 is not significantly ($P > 0.05$) different from that observed previously (Table I). However, for the experiments reported here, the duration of phase 1 (19 ± 1.3 s) was significantly ($P < 0.01$) prolonged, whereas the duration of phase 2 (11 ± 1.2 s) was significantly ($P < 0.01$) shortened (Table I). The amplitudes of I_{sm} (-0.26 ± 0.030) and I_p (1.6 ± 0.29 nA) were not significantly different than observed previously (Table I), whereas the amplitude of I_{on} was significantly ($P < 0.025$) smaller than observed previously (Table II).

Membrane patch currents recorded in eggs clamped between -30 and 0 mV. For eggs clamped between -30 and 0 mV, the currents measured from the membrane patch (Fig. 3B), which includes the site of sperm attachment, duplicated (Fig. 4) the simultaneously recorded whole egg currents (Figs. 3A and 4) at I_{on} and for most of

TABLE III
*Amplitudes of Type I Activation Currents for the Whole Egg and the Membrane Patch
 Clamped at Membrane Potentials between -90 and -60 mV
 (Mean = -78 ± 2.5 mV)*

	I_{on}	I_{sm}	I_p
	nA	nA	nA
Whole egg, sperm in pipette	$-0.12 \pm 0.043^*$ ($n = 4$)	-0.31 ± 0.11^f ($n = 4$)	-2.6 ± 0.51 ($n = 3$)
Membrane patch	-0.12 ± 0.043 ($n = 4$)	-0.38 ± 0.19 ($n = 4$)	n.a.
Membrane patch, corrected	$-0.14 \pm 0.045^*$ ($n = 4$)	-0.45 ± 0.24^f ($n = 4$)	n.a.

Values with the same superscript were not significantly different ($P > 0.05$) using analysis of variance.

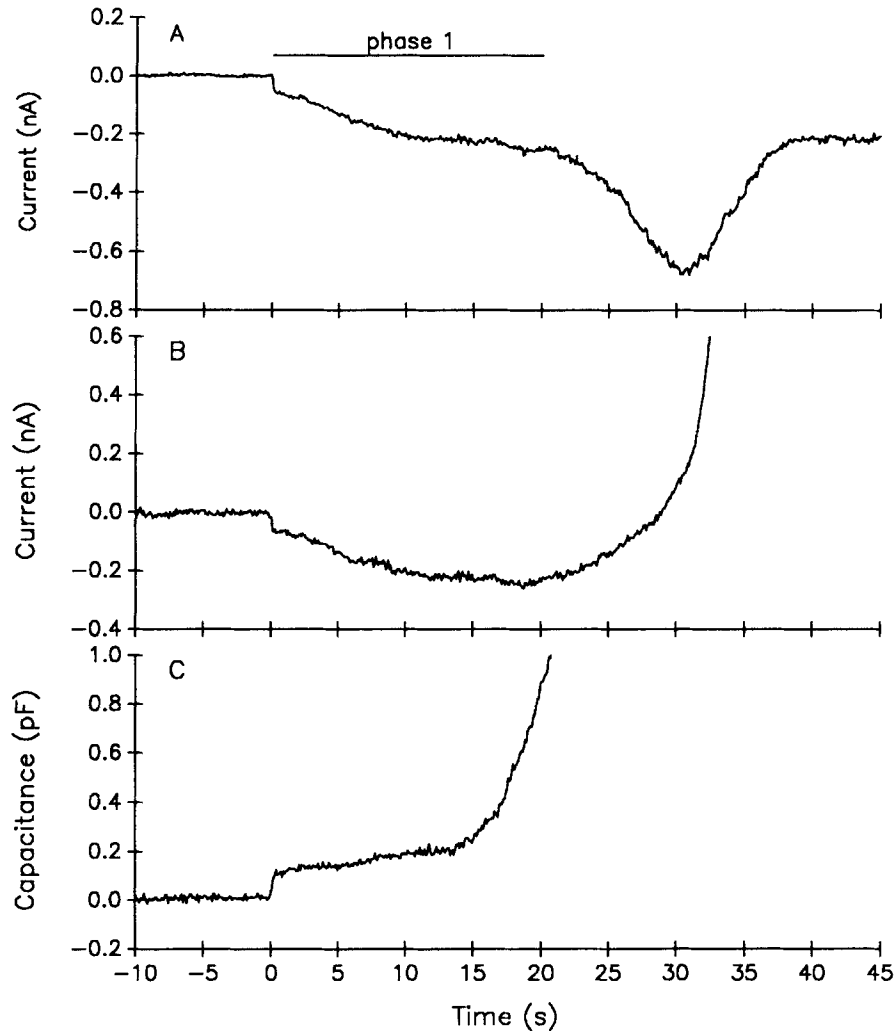


FIGURE 3. Simultaneously recorded whole egg current (*A*), membrane patch current (*B*), and membrane patch capacitance (*C*) during a type I activation current after insemination by introduction of sperm into the interior of the loose patch clamp pipette. The membrane potential of the egg was maintained at -10 mV by the whole egg voltage clamp. I_{on} occurs at 0 s. The duration of phase 1 is indicated by a horizontal line above the current trace in *A*. Phase 1 ends at I_{sm} as phase 2 begins as a more rapid increase of inward current. Phase 2 ends and phase 3 begins at the time of I_p , the maximum inward current, at 31 s in *A*. The membrane patch current (*B*), low pass filtered to remove the sinusoidal currents, duplicates the whole egg current (*A*) throughout the first 18.8 s of the activation current (also see Fig. 4). Increases of the capacitance of the membrane patch (*C*) occur abruptly at the time of I_{on} (0 s) and a second time rising smoothly beginning between 5 and 18.8 s after I_{on} . The egg from which these records were obtained subsequently cleaved to form a swimming blastula, indicating that sperm entry occurred.

the duration of phase 1. Thereafter, the two traces started to diverge; i.e., in Figs. 3 B and 4, the inward membrane patch current started to decrease while the whole egg current continued to increase. Divergence of the traces may be related to deterioration of the seal between the tip of the patch clamp pipette and the surface of the egg (McCulloh and Chambers, 1991). Therefore, a conservative estimate is that appreciable deterioration of the seal resistance had not occurred during the period from I_{on} to the time when the two traces started to diverge.

Measurements of the membrane patch currents were discontinued after the start of phase 2 in most experiments due to changes of the seal resistance (McCulloh and Chambers, 1991), which were not measured.

The amplitude of I_{on} measured for the membrane patch was -0.05 ± 0.011 nA ($n = 10$). To compare this value with the amplitude of I_{on} measured for the whole egg (Table II) a correction needs to be applied to the measured membrane patch currents to account for the loss of current through the seal resistance. The fraction of the membrane current expected to be measured by the loose patch clamp can be estimated for each egg by dividing the seal resistance by the sum of seal resistance

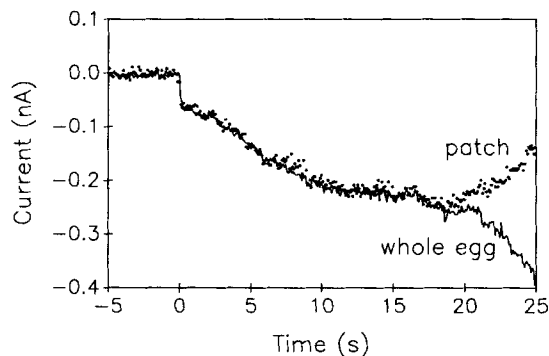


FIGURE 4. Superimposition of whole egg current (solid line) and membrane patch current (closed circles) for the first 25 s of the same type I activation current shown in Fig. 3. Note the similarity of the traces between the time of I_{on} (0 s) and the time that the traces diverge at 18.8 s.

plus pipette resistance (0.81 ± 0.12 ; $n = 10$). Dividing each patch's value for the measured membrane patch current by its own fraction gives the corrected value of -0.06 ± 0.013 nA. This value is not significantly different from the amplitude of I_{on} measured for the whole egg ($P > 0.05$). Since the two traces increased in parallel from I_{on} to the point of divergence of the two traces, we conclude that throughout this period the amplitudes of the currents for the whole egg and for the membrane patch (as corrected) are the same. Even at the end of phase 1 measured for the whole egg, 19 ± 1.3 s after I_{on} , or only 3.2 s after the two traces started to diverge, the corrected value for the membrane patch current was not significantly different from the whole egg current at I_{sm} . We conclude, therefore, that all of the whole egg current during phase 1 can be accounted for by currents flowing through the membrane patch. Moreover, since the fertilizing sperm attached to the membrane patch, we conclude that the current during phase 1 of type I activation currents is localized to the site, or the immediate vicinity, of the attached sperm.

Whole egg and membrane patch currents recorded simultaneously during type I activation currents in eggs clamped between -90 and -60 mV. Currents recorded from

the whole egg maintained within this range of potentials during insemination with sperm from within the patch clamp pipette are summarized in Table III. Except for the greater amplitude of the currents, the results again show the similarity in the amplitudes of the whole egg current during phase 1 and the membrane patch currents, as described earlier for eggs clamped at membrane potentials between -30 and 0 mV.

Conductance changes of the whole egg clamped between -30 and 0 mV and of the membrane patch measured simultaneously. Membrane conductance for the whole egg was measured in three eggs by applying voltage pulses ~ 1 s in duration with the single-microelectrode, switched voltage clamp and noting the amplitude of the resultant whole egg current pulses (Fig. 5 A). Membrane conductance (Fig. 5 B) increased concomitantly with the inward currents averaging an increase of 26 ± 4.0 nS ($n = 3$) at I_{sm} and 140 ± 40 nS ($n = 3$) at I_p .

The conductance of the membrane patch surrounding (and including) the site of sperm-egg attachment was measured in the same three eggs by noting the membrane patch current steps (Fig. 5 C) in response to voltage pulses applied to the whole egg. The conductance of the membrane patch surrounding (and including) the site of sperm-egg attachment increased abruptly at the time of I_{on} and increased slowly over the subsequent seconds (Fig. 5 D) simultaneously and in parallel with the increase of conductance for the whole egg (Fig. 5 B). The values calculated for the conductance increases of the membrane patch and the whole egg during phase 1 are not significantly different, suggesting that the entirety of the membrane conductance increase observed for the whole egg was localized to the membrane patch. These results verify our finding, described earlier, that all of the whole egg current during phase 1 can be accounted for by currents flowing through the membrane patch and/or the seal. However, during phase 2 the values for the conductance increases of the membrane patch and of the whole egg are quite different. The likely sources of this difference are the occurrence of conductance increase over regions of the egg other than the membrane patch and/or changes in the seal resistance of the loose patch clamp pipette, which was not monitored in these experiments.

Capacitance of the membrane patch during type I responses in eggs clamped at potentials between -90 and 0 mV. The rationale for these experiments is that when electrical continuity is established between the cytoplasm of the sperm and egg due to gamete membrane fusion, this event can be recorded as an increase in capacitance of the membrane patch due to addition of the surface area of the sperm to that of the patch at the egg's surface.

Capacitance of the membrane patch that surrounds and includes the site of sperm attachment and sperm-egg interaction was measured by monitoring the imaginary component of admittance of the patch, reported here in picofarads, units of capacitance. The validity of this conversion from the imaginary component of admittance to units of membrane capacitance is discussed below.

Two capacitance increases occurred during phase 1 of the type I activation current recorded in eggs held at membrane potentials between -90 and 0 mV (Fig. 3 C): (a) an abrupt, nearly step-like increase of 0.22 ± 0.028 pF ($n = 15$) occurring at the same time as I_{on} ; and (b) a slower rise of capacitance beginning 8.7 ± 0.87 s ($n = 11$) after I_{on} . The limit of time resolution for the capacitance measurement was ~ 30 ms due to filtering, and the abrupt increases of capacitance and of inward current always

occurred within 30 ms of each other. The second rise of capacitance began 8 ± 1.8 s ($n = 6$) before divergence of the currents measured with the loose patch clamp and the single-microelectrode, switched voltage clamp. However, the increase was not completed by the time of divergence of currents, suggestive of changes in R_{seal} . Therefore, no quantitative measure of the magnitude of the increase is reported here except to say that the capacitance increase at the time of divergence had roughly doubled the increase at the time of I_{on} .

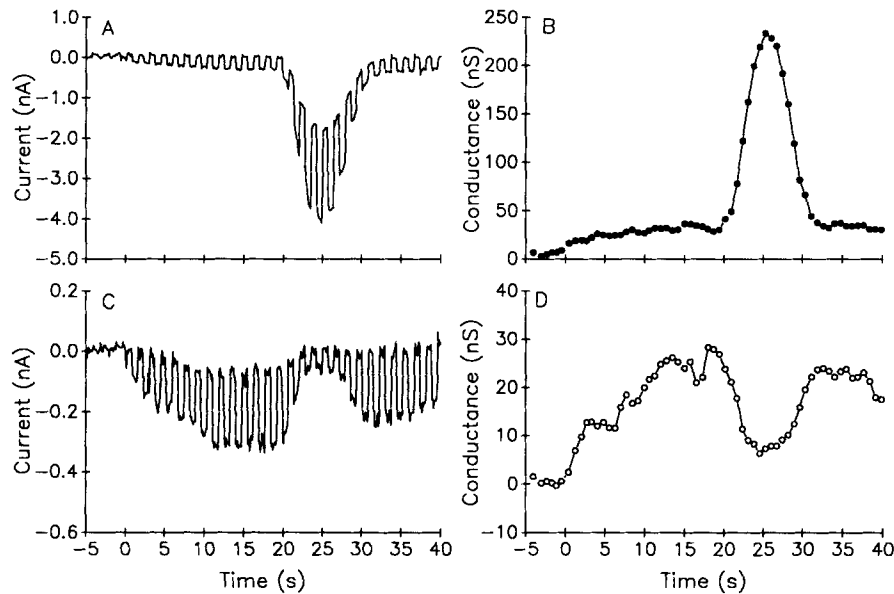


FIGURE 5. Currents (*A, C*) and conductances (*B, D*) for the whole egg (*A, B*) and the membrane patch (*C, D*) during a type I activation current after insemination by introduction of sperm into the interior of the loose patch clamp pipette. The membrane potential of the egg was stepped from -10 to 0 mV and back to -10 mV roughly once every 1.45 s. The amplitudes of steps in the current trace for the whole egg (*A*) indicate the conductance of the membrane for the whole egg (*B*). The amplitudes of current steps visible in the current trace for the membrane patch (*C*) are likewise indicative of the conductance for the membrane patch (*D*). I_{on} occurs at 0 s; I_{sm} occurs at 19 s; I_{p} occurs at 25 s. The membrane patch current and conductance are indistinguishable from the whole egg current and conductance, respectively, during phase 1 (0–19 s) of the activation current (after subtraction of whole egg leakage current and conductance).

The increase of capacitance for the membrane patch at the time of I_{on} in eggs maintained between -90 and -60 mV (0.17 ± 0.047 pF, $n = 4$) was not significantly different ($P > 0.05$) from the increase of capacitance observed in eggs maintained at potentials between -30 and 0 mV (0.27 ± 0.050 pF, $n = 7$).

Membrane capacitance for the membrane patch during type I activation currents for which eggs were fertilized by sperm outside the loose patch clamp pipette. Capacitance measurements were attempted as control experiments in which it was certain that the fertilizing sperm was not within the pipette. These were performed while the whole

egg's membrane potential was maintained at -10 mV. Sperm were added to the bath, outside the pipette, using 8 of the 18 experiments for which sperm were added to the bath after failure to find sperm in the polyethylene tube. The capacitance of the membrane patch never changed at the time of I_{on} , in contrast to what was observed with sperm inside the pipette. We conclude that the abrupt increase of capacitance for the membrane patch at the time of I_{on} requires the presence of sperm within the pipette, and that the capacitance increase cannot be explained as the consequence of electrical cross-talk between the two voltage clamps.

The imaginary component of admittance for the membrane patch did change during phase 2 when sperm were added outside the pipette. The change was neither simple nor reproducible, increasing in some cases and decreasing in others and seldom changing in a monotonic fashion. We chose not to pursue these measurements any further.

Justification of equating C_m with the imaginary component of admittance. The amplitude of the imaginary component of current corresponded to an increase of admittance of ~ 1 nS, whereas the increase of the real admittance was ~ 5 – 10 nS at the time of I_{on} , ~ 5 – 10 times as large. To affirm that the imaginary component measured was truly the imaginary component, and not simply a projection of the real component onto the selected phase angle, measurements in different eggs were made at different phase angles between -100 and -80° . Within this range of phase angles, the real component should be reduced to a value near 0 and change sign. Therefore, if the values obtained resulted solely from small spurious projections of the real component onto the selected phase angle, then the measured values would have been significantly reduced in amplitude or would have changed sign over this range of phase angles. The measured imaginary components did not change sign and thus must have involved a true imaginary component. This conclusion is supported by the finding that the conductance increases (inferred from the measured currents) were roughly twice as large between -90 and -60 mV as at potentials between 0 and -30 mV, and yet the capacitance increases were the same.

Up to this point, we have considered that increases of the imaginary component of admittance are caused solely by increases of the membrane capacitance. However, the amplitude of the imaginary component is determined by several circuit elements. Now we will systematically consider all the circuit elements G , G_s , and C_m (Fig. 1) included in Eq. 2 describing the imaginary component of the complex admittance.

G consists of the conductance for the membrane patch in parallel with the seal conductance. Increases of conductance for the membrane patch during phase 1, up to the time of divergence of currents at 16 s (≤ 20 nS), would not significantly affect measurements of the complex admittance in response to changes of potential in the loose patch clamp pipette because G remains dominated (~ 25 times) by the large seal conductance (~ 500 nS) in parallel with a small membrane conductance throughout this period. The increase of conductance at I_{on} would result in a decrease in the imaginary component of admittance. Therefore, the observed increase of membrane conductance at the time of I_{on} could not result in the significant changes of the imaginary component that were observed.

The failure of divergence for the currents measured with the loose patch clamp

pipette and the single-microelectrode, switched voltage clamp suggests that after I_{on} , significant deterioration of the R_{seal} occurs only very late during phase 1, nearing the time of I_{sm} . Therefore, deterioration of R_{seal} could not account for the increase of the imaginary component of admittance at the time of I_{on} . Furthermore, an increase of seal conductance (decrease of R_{seal}) would lead to a decrease of the imaginary component (see Eq. 2) and therefore could not account for the observed increase of the imaginary component.

A decrease of the seal conductance (increase of R_{seal}), concomitant with I_{on} , is a possibility that cannot be completely dispelled because it would be difficult to resolve using our techniques. However, loose patch clamp measurements of the current during I_{on} and throughout most of phase 1 account for all the current flowing into the egg during this period. This was concluded because whole egg current amplitudes were not significantly different from the membrane patch currents, taking into account the seal and pipette resistances measured before insemination. The similarity of these values suggests that the seal resistance does not change dramatically or abruptly during I_{on} . A systematic decrease of the seal conductance is described for loose patch clamp experiments performed using eggs of this species but involving sperm addition to the bath (McCulloh and Chambers, 1991). In that study, the decreased seal conductance occurred in association with the exocytosis of cortical granules, attaining its minimum ~ 8 s before the onset of deterioration of seal resistance. The seal resistance gradually increased to ~ 1.7 times the value before insemination. Such an increase would just barely account for the magnitude of the imaginary component but is neither likely to occur at the appropriate time nor occurs as abruptly as the initial increase of the imaginary component described in this paper. In addition, the increase of the imaginary component of admittance occurs even in the absence of activation or phase 2 phenomena during type II sperm transient currents (see below).

We consider it unlikely that the series conductance (G_s) attributable to $1/R_{pip}$ suddenly increases concomitantly with I_{on} , because the sudden presence of the sperm would be more likely to cause a decrease of the series conductance due to a small reduction of the cross-sectional area of the tip of pipette at its most resistive point. However, we cannot formally rule this option out.

Without reason to believe either that G decreases abruptly or that G_s increases abruptly during phase I, we are compelled to believe that the abrupt increase of the imaginary component coincident with I_{on} is caused by an increase of C_m , the membrane capacitance, a change that is expected to occur at some time during phase I.

Current and Capacitance Measurements for Eggs Inseminated from Within the Loose Patch Clamp Pipette, Where Sperm Entry Was Precluded by Adjustment of the Membrane Potential

Measurements where sperm entry failed to occur due to maintenance of the membrane potential between -90 and -60 mV (type II and III responses). To investigate the inhibitory effect of large, negative membrane potentials on sperm entry, we maintained the whole egg membrane potential at values between -90 and -60 mV using the single-microelectrode, switched voltage clamp, while the loose patch clamp monitored currents and

capacitance for the membrane patch surrounding (and including) the site of sperm–egg attachment. Within this membrane potential range, several types of electrophysiological responses occur (Lynn et al., 1988): type I activation currents associated with sperm entry, type II sperm transient currents associated with failure of sperm entry, and type III modified activation currents in which the egg is activated but sperm entry fails. Sperm inside the patch clamp pipette caused 17 responses in 14 successful eggs of the 21 eggs attempted. Type I activation currents leading to sperm entry occurred for six of the responses (described above). Sperm transient currents occurred for eight of the responses. Type III modified activation currents occurred three times.

Sperm Transient Currents (Type II Responses): The sperm transient currents measured with the single-microelectrode, switched voltage clamp (Fig. 6A) for eggs interacting with sperm from within the loose patch clamp pipette initiated with an abrupt, inward current (I_{on}) averaging -0.5 ± 0.12 nA ($n = 6$) in amplitude, increasing to a maximum (I_m) of -1.5 ± 0.46 nA ($n = 6$) (Table IV). A characteristic feature of sperm transient currents is that they abruptly decrease in amplitude, returning to the baseline current 7 ± 3.7 s ($n = 6$) after I_{on} . The currents monitored using the loose patch clamp (Fig. 6B) duplicated the whole egg currents recorded simultaneously, and initiated with an abrupt inward current (I_{on}) averaging -0.5 ± 0.11 nA ($n = 6$) that increased to a maximum of -1.3 ± 0.40 nA ($n = 6$) (Table IV). To compare these amplitudes with the corresponding values measured for the whole egg, a correction must be applied to the membrane patch currents, as previously discussed in relation to the phase 1 currents of type I responses. The fraction of current expected to be measured by the loose patch clamp pipette is the seal resistance divided by the sum of the seal resistance plus the pipette resistance, which amounts to 0.88 ± 0.011 ($n = 6$). Dividing the measured values of membrane patch current by the fraction calculated in the same egg gives the corrected amplitudes for the membrane patch currents (see Table IV). The similarity between the whole egg currents and the corrected membrane patch currents (Table IV) indicates that all the current measured for the whole egg throughout the duration of the sperm transient current can be accounted for by current flowing into the egg from the loose patch clamp pipette plus current through the seal resistance; therefore, the current was localized to the membrane patch that surrounds as well as includes the site of sperm–egg attachment. The seal resistance did not change during and after the sperm transient current as long as a type I or type III activation response did not intervene.

Capacitance was monitored for the membrane patch using the lock-in amplifier to obtain the imaginary component of the sinusoidal currents in response to a sine wave command voltage applied to the loose patch clamp. Membrane capacitance (Fig. 6C) increased abruptly by 0.14 ± 0.011 pF ($n = 5$) at the time of I_{on} . The capacitance trace rapidly returned to the baseline capacitance level at the same time that the sperm transient current rapidly returned to its baseline current level. During the intervening period between I_{on} and the return of current to the baseline level, the capacitance trace increased and decreased in parallel with the current traces (Fig. 6). Other than the small fluctuations of capacitance in parallel with membrane currents, no further increase of capacitance was observed. The amplitude of the abrupt

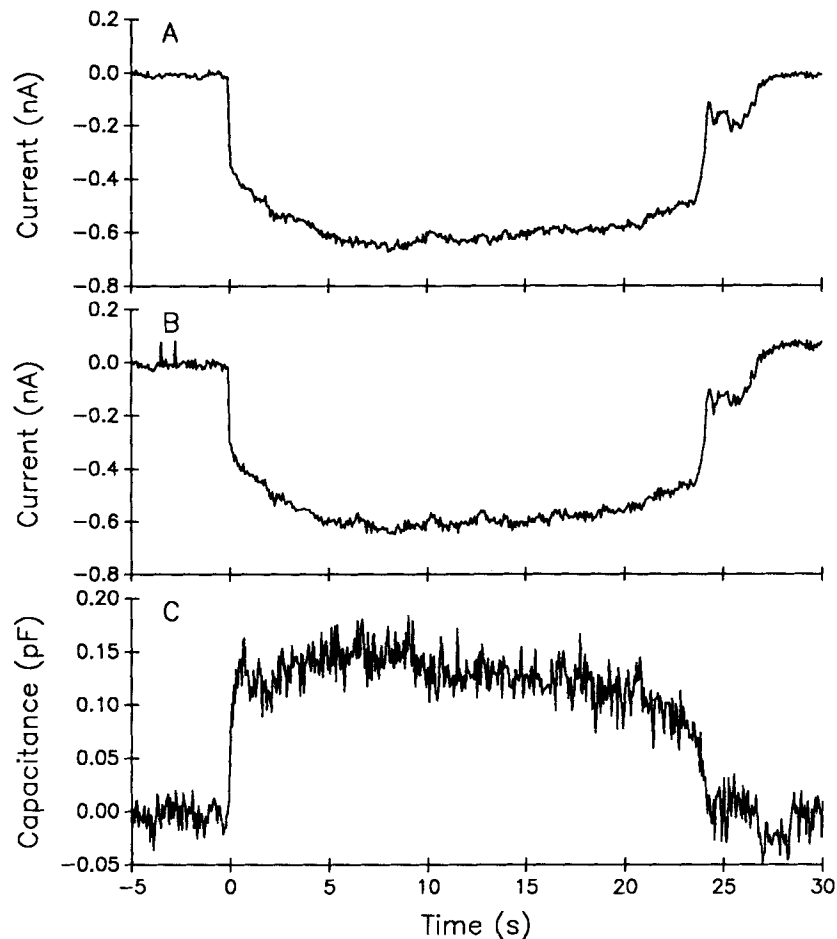


FIGURE 6. Simultaneously recorded whole egg current (*A*), membrane patch current (*B*), and membrane patch capacitance (*C*) during a type II sperm transient current that occurred after insemination by introduction of sperm into the interior of the loose patch clamp pipette. The membrane potential of the egg was maintained at -85 mV throughout the period of recording. The whole egg current (*A*) initiates with an abrupt inward current I_{on} at 0 s and remains elevated for a period, which is uncharacteristically long. Beginning at 23.5 s, the whole egg current (*A*) rapidly cut off in at least two steps. The membrane patch current (*B*), low pass filtered to remove the sinusoidal currents, duplicated the entire whole egg response (*A*). The capacitance of the membrane patch (*C*) increased abruptly at the time of I_{on} and cut off rapidly in two steps mirroring both the whole egg current (*A*) and the membrane patch current (*B*). The egg from which these records were obtained failed to form a sperm aster and did not cleave, indicating that the sperm failed to enter.

increase of capacitance during type II sperm transient currents was not significantly different ($P > 0.05$) from the amplitude of the abrupt increases of capacitance observed in eggs during type I activation currents at potentials between -90 and -60 mV or at potentials between -30 and 0 mV. However, the capacitance profile

for type II sperm transient currents differs from the capacitance changes observed during the phase I currents of type I responses previously described, in that for the sperm transient currents the capacitance rapidly returns to baseline and lacks a second increase of capacitance.

When sperm transient currents occurred, the egg otherwise remained in the unfertilized state (i.e., the fertilization envelope did not elevate, the sperm aster did not form, and the egg failed to cleave). Like the rapid cutoff of current that characterizes a sperm transient, the return of capacitance to baseline signifies that the sperm will not enter the egg.

Modified Activation Currents (Type III Responses): Modified activation currents were characterized by initiating with simultaneous abrupt, inward currents (I_{on} 's) averaging -0.3 ± 0.13 nA ($n = 3$) for the whole egg and -0.2 ± 0.16 ($n = 2$) for the membrane patch. Currents increased to -0.7 ± 0.27 nA ($n = 3$) for the whole egg and to -1 nA ($n = 1$) for the membrane patch before simultaneous, rapid decreases

TABLE IV
Currents for the Whole Egg and the Membrane Patch during Type II and Phase I of Type III Responses in Eggs Maintained at Potentials between -90 and -60 mV
(Mean = -84 ± 0.8 mV)

	I_{on}	I_m
	nA	nA
Type II responses		
Whole egg	$-0.5 \pm 0.12^*$ ($n = 6$)	-1.5 ± 0.46^i ($n = 6$)
Membrane patch	-0.5 ± 0.11 ($n = 6$)	-1.3 ± 0.40 ($n = 6$)
Membrane patch, corrected	$-0.5 \pm 0.12^*$ ($n = 6$)	-1.5 ± 0.46^i ($n = 6$)
Phase I of type III responses		
Whole egg	-0.3 ± 0.13^j ($n = 3$)	-0.7 ± 0.27^f ($n = 3$)
Membrane patch	-0.2 ± 0.16 ($n = 2$)	-1.0 ($n = 1$)
Membrane patch, corrected	-0.3 ± 0.18^g ($n = 2$)	-1.1^h ($n = 1$)

Values bearing the same superscript were not significantly different ($P > 0.05$) using analysis of variance.

of current 15 ± 3.1 s ($n = 3$) after I_{on} . The feature that distinguishes a modified activation current from a sperm transient current is the presence of a second phase of inward current after the rapid decrease of current that terminates phase I. The simultaneity of whole egg and patch currents as well as their indistinguishable amplitudes indicate that during the first phase, the whole egg current is localized to the membrane patch that surrounds and includes the site of sperm-egg attachment. The peak (I_p) of the second phase of inward current attained a value of -2 nA ($n = 1$), 52 ± 6.5 s ($n = 2$) after the initial I_{on} . Near the time that the second phase of the response began, the membrane patch currents deviated from the whole egg currents. The likely explanations for this deviation are conductance increases elsewhere than in the region near the sperm and/or deterioration of the seal resistance for the loose patch clamp pipette. Recordings with the loose patch clamp were not analyzed beyond this time.

The capacitance of the membrane patch surrounding and including the site of sperm attachment was monitored using lock-in amplifier analysis of the sinusoidal currents in response to the sine wave command voltage applied to the loose patch clamp. The capacitance profiles amalgamated characteristics of both the type I and type II responses. The capacitance of the membrane patch increased 0.2 ± 0.067 pF ($n = 2$) at the time of I_{on} (similar to type I and II responses), remained at this level for several seconds after I_{on} , then started a gradual smooth increase (similar to type I responses). However, the capacitance decreased rapidly at the same time as the rapid decrease of current terminating phase 1 (similar to type II responses). After the rapid decrease of capacitance, the gradual smooth increase continued although the amplitude of this change is in question because the resistance of the seal was not known at this time. Elevation of the fertilization envelope accompanied phases 2 and 3. These eggs exhibited no asters and did not divide, indicating that sperm had failed to enter.

For both sperm transient currents and modified activation currents, the rapid decrease of capacitance that occurred coincidentally with the rapid decrease of current indicated that the sperm would not enter the egg (Table V). Nonetheless, the prior

TABLE V
Association of Capacitance Changes with Sperm Entry

	Sperm entry*	No sperm entry
Abrupt, maintained increase of C_m	9	0
Abrupt increase and rapid decrease of C_m	0	6
No increase of C_m	0	5 [†]

*The presence of a sperm aster was the criterion used to determine if the sperm had entered.

[†]Number of eggs exposed to sperm at +35 mV. All other entries sum the number of sperm that caused electrophysiological responses.

occurrence of the abrupt increase of capacitance simultaneously with I_{on} indicated that electrical continuity between the gametes had taken place. Consequently, the failure of sperm entry occurred after electrical continuity had been established.

Absence of electrophysiological changes after sperm attachment in eggs maintained at membrane potentials between +25 and +45 mV. Eggs were inseminated by introduction of sperm into the patch clamp pipette while the the membrane potential of eggs was maintained constant at +35 mV with the single-microelectrode, switched voltage clamp throughout insemination by sperm inside the pipette. Simultaneously, the potential across the membrane patch was varied as a 1 kHz sine wave between +25 and +45 (20 mV peak to peak). Successful sperm additions that resulted in sperm swimming and colliding with the membrane patch occurred seven times in 13 eggs attempted at this potential. Once a sperm had collided with the membrane patch, the command potential for the whole egg's voltage clamp was maintained at +35 mV for an additional 20 min (roughly twice the time constant describing the exponential distribution of waiting times in Fig. 2). No current or capacitance responses were observed during the 20-min period (no figure shown). After the 20-min exposure at +35 mV, the command potential for the whole egg's voltage clamp was shifted to a

value near -10 mV for several minutes. On two occasions, type I activation currents and capacitance increases occurred as described above, but only after the shift of membrane potential near to -10 mV. Statistical arguments indicate that the failure to establish electrical continuity at positive membrane potentials is significant.²

Association of Sperm Entry with the Initial Abrupt Increase of Capacitance and Its Maintenance

Sperm entry occurred only in those eggs where a step-like increase of the capacitance for the membrane patch occurred during sperm-egg interaction, and where the increase in capacitance was maintained (only for type I activation currents; see Table V). The probability that the appearance of an aster and the sustained abrupt increase of capacitance occurred independently was $P < 0.005$ (contingency likelihood test, \ln [likelihood ratio] > 12 , with two degrees of freedom).

Association of the Exocytosis of Cortical Granules with the Second, Smooth Increase of Capacitance

The second, smooth increase of capacitance for the patch, observed beginning either during the sustained increase of capacitance (type I activation currents) or before the rapid decrease of capacitance (type III, modified, activation currents), was associated with elevation of the fertilization envelope (Table VI). Elevation of the fertilization envelope indicates that exocytosis of cortical granules has occurred (Moser, 1939) and that the egg has been activated. A second, smooth increase of capacitance for the patch was never observed in association with type II sperm transient currents. These are not accompanied by elevation of the fertilization envelope. Unless elevation of the fertilization envelope occurs, the smooth increase of capacitance for the patch is never observed, and conversely, elevation of the fertilization envelope never occurred without a concomitant smooth increase of the capacitance (Table VI). The probability that elevation of the fertilization envelope and the second, smooth increase of capacitance occurred independently was $P < 0.005$ (contingency likelihood test, \ln

²The probability that an egg does not undergo an electrophysiological response before 20 min after sperm addition is estimated by the area under the tail of the characteristic probability density function that is approximated by the exponential distribution of waiting times (Fig. 2) integrating between 20 min and infinite time. The probability that an egg maintained at a membrane potential of -90 to 0 mV did not undergo an electrophysiological response within the first 20 min of sperm exposure, using the time constant obtained by fitting (Fig. 2), is 0.132 for one egg. The probability that this occurs for n eggs is 0.132^n . Therefore, the probability that no capacitance increase or current response occurred solely by chance for seven eggs while the whole egg's membrane potential was maintained at $+35$ mV for 20 min was $P \leq 7 \times 10^{-7}$. If we consider only those two eggs that exhibited responses when their membrane potential was returned to a value near -10 mV, the probability that they did not undergo capacitance increases at potentials between $+25$ and $+45$ mV is $P \leq 0.132^2 = 0.0174$. Since the probability that a capacitance increase failed to occur by chance was < 0.05 , we conclude that membrane potentials between $+25$ and $+45$ mV significantly preclude the increases of capacitance and inward current by blocking sperm-egg interaction at a step before establishment of electrical continuity. Therefore, sperm-egg membrane fusion is precluded at these positive potentials. (Note that Fig. 2 was obtained by measuring time from the time of sperm addition to the seawater in the pipette, whereas in these experiments we have been more conservative, measuring from the time of the first sperm-egg collision, which generally occurs several minutes after introduction of sperm to the seawater inside the pipette.)

[likelihood ratio] > 7, with one degree of freedom). Therefore, elevation of the fertilization envelope was obligatorily interrelated to the second, smooth increase of capacitance. An increase of surface area of the egg's plasma membrane due to exocytosis of the cortical granules (Jaffe, Hagiwara, and Kado, 1978; Kado, 1978; McCulloh, 1985) in the membrane patch (in the vicinity of the sperm) is the likely explanation for the second, smooth increase of capacitance for the membrane patch.

DISCUSSION

Sperm introduced into the inside of a loose patch clamp pipette that was pushed against the surface of an egg were able to activate and enter the egg as well as to evoke activation currents similar to those caused by sperm in the bath. All the whole egg current during the first approximately four-fifths of phase 1 of type I and III activation currents, and the entirety of the type II or sperm transient currents was accounted for by currents through the membrane patch. For the three types of responses, a step-like increase of capacitance for the membrane patch occurred coincidentally with I_{on} (irrespective of whether the sperm entered or not). A second, smooth increase of capacitance occurred during the later part of phase 1 for type I and III activation currents, which was associated with exocytosis of cortical granules.

TABLE VI
Association of the Second Increase of Capacitance with the Exocytosis of Cortical Granules

	Fertilization envelope* elevated	Fertilization envelope did not elevate
Second increase of C_m	12	0
No second increase of C_m	0	6

*Elevation of the fertilization envelope served as an indication of exocytosis of cortical granules.

When the egg's membrane was maintained at negative potentials that precluded sperm entry (type II and III responses), the initial step-like increases of both capacitance and current were followed by a rapid decrease of capacitance toward the baseline, coincident with cutoff of the inward current. When the egg's membrane was maintained at positive potentials that precluded sperm entry, no indication of increase in capacitance was detected and no abrupt or any other change in the baseline current was observed.

Localization of Ion Channels

Localization of channels to the site of sperm attachment for phase 1 of type I and III activation currents, and for type II sperm transient currents. Sperm that fertilized the egg had access only to the egg's plasma membrane patch circumscribed by the tip of the pipette since the sperm were introduced only inside the loose patch clamp pipette. The currents through the membrane patch and the whole egg (as well as the membrane conductances) were indistinguishable for the first 16 s of phase 1 (total duration of phase 1 = 19 s) during type I activation currents, during type II sperm transient currents, and during phase 1 of type III modified activation currents. This indicates

that the channels responsible for the inward current (and the increase of conductance) during phase 1 and the sperm transient currents are localized to the membrane patch surrounding and including the site of sperm attachment. These direct, quantitative observations that the channels are localized to, or in the vicinity of, the attached sperm are in agreement with our earlier suggestion based on our failure to detect increases of conductance for the membrane patch during phase 1 when sperm were added to the bath outside the pipette (McCulloh and Chambers, 1991).

The pipette was placed at a location on the surface of the egg with no prior knowledge of any preferred site of sperm entry because no signs of polarity remain after removal of the jelly coats (Schroeder, 1980). For eggs clamped at potentials more negative than 0 mV, in almost every case where sperm swam after their introduction into the pipette, at least one sperm interacted with the egg, evoking activation currents and fertilization envelope elevation essentially indistinguishable from those observed when sperm were added to the bath. These data indicate that occurrence of responses does not depend on the site selected for placement of the loose patch clamp pipette and that the entire surface of the egg is capable of interacting with sperm in a manner that can lead to fertilization. We cannot discount the possibility that a particular region of the egg's surface is more susceptible, because quantitative probabilities of sperm-egg interaction were not determined.

Currents during phase 2 involve the entire surface of the egg. When eggs were inseminated by introducing sperm into the patch clamp pipette, phase 2 of type I (whole egg) activation currents were briefer, lasting approximately half as long, compared with eggs inseminated by adding sperm to the bath (Table I). Fertilizing sperm when introduced into the patch clamp pipette, as well as when added to the bath, activate an egg from the sperm's site of attachment at the surface of the egg. However, in the former case, the small region of the egg's surface to which the sperm attaches is pushed deep into the egg to a position near the egg's center. This difference in geometry caused by indenting the egg's surface may account for the difference in the duration of phase 2. Using the patch clamp technique, but inseminating the eggs by addition of sperm to the bath, we have shown that during phase 2 a localized band-shaped zone of the egg's membrane undergoes a transient increase in membrane conductance that progresses over the surface of the egg from the site of sperm-egg contact to the opposite pole of the egg. Progression of the wavefront of increased membrane conductance is caused by a cytoplasmic message that travels from the site of sperm attachment throughout the cytoplasm of the egg (McCulloh and Chambers, 1991). When the egg is inseminated from the bath, the cytoplasmic message, which induces the wave of increased conductance to sweep over the surface of the egg, must traverse the entire diameter of the egg (from one pole to the antipode). On the other hand, when the sperm is introduced into the patch pipette, the cytoplasmic message travels from the site of sperm attachment near the center of the egg along the egg's radii to cause an increase of membrane conductance over most of the egg's surface nearly coincidentally. Consequently, the duration of the conductance increase would be shorter (reflecting the time course for channel opening and closing) without the prolongation caused by propagation across the entire egg from one pole to the antipode.

When Does the Sperm Fuse with the Egg?

The abrupt increase of capacitance for the membrane patch simultaneously with I_{on} is the consequence of the establishment of electrical continuity between the sperm and the egg. We conclude from our data that the step-like increase in capacitance of the patch at I_{on} results from establishment of electrical continuity between the cytoplasm of the sperm and the egg (i.e., at I_{on} the capacitance of the sperm's membrane becomes accessible for measurement). The step-like increase in capacitance at I_{on} occurs when, and only when, the sperm elicits an electrophysiological response (type I, II, or III), irrespective of whether or not sperm entry ensues. The sperm is clearly responsible for the step-like increase in capacitance of the patch, since the increase is seen only when the egg is inseminated by addition of sperm to the patch pipette. Moreover, attachment of the sperm to the surface of the egg alone cannot account for the step-like increase of capacitance of the patch, since for eggs clamped at positive membrane potentials, the sperm attach both readily and tenaciously to the egg surface, but neither cause an increase in capacitance nor elicit an electrophysiological response.³

We know from earlier studies on echinoderm eggs that sperm entry is preceded by fusion of the gametes' membranes (Colwin and Colwin, 1967). Electrical continuity between the gametes must accompany this process. Consequently, it is particularly significant that sperm entry (always associated with a type I activation current) is never observed unless preceded by a step-like increase of capacitance for the patch.

The surface area of the sperm is of the order of magnitude that would be expected if the step-like increase in capacitance at I_{on} is the consequence of establishment of electrical continuity between the cytoplasm of the sperm and egg. From measurements of *L. variegatus* sperm viewed by scanning electron microscopy, the surface of the sperm's head was estimated at $16 \mu\text{m}^2$ (approximated as a cone $6 \mu\text{m}$ in height and $1.5 \mu\text{m}$ in diameter at its base), and the tail at $70 \mu\text{m}^2$ (approximated as a cylinder $70 \mu\text{m}$ in length and $0.3 \mu\text{m}$ in diameter). If the entire membrane of the sperm were accessible for capacitance measurements, on the basis of $1 \mu\text{F}/\text{cm}^2$ of surface area (Cole, 1972), the capacitance of the sperm's head would amount to 0.16 pF, and the tail to 0.70 pF, for a total of 0.86 pF. Before a comparison can be made between these values and the measured increase of capacitance for the patch at the time of I_{on} of ~ 0.22 pF, the intracellular resistance of the sperm must be taken into consideration. If the assumption is made that the specific electrical resistance of the sperm's cytoplasm is $\sim 200 \Omega \text{ cm}$, the same as that of the egg (adapted from Cole and Curtis, 1938; the value for the sperm's cytoplasm is unknown), it can be estimated that the resistance to passage of current through the entire length of the tail is $\sim 1.9 \text{ G}\Omega$ or $28 \text{ M}\Omega/\mu\text{m}$. Consequently, due to the high resistance to passage of current along the length of the sperm's tail, much of the tail would be inaccessible for measurement of the capacitance. It is, therefore, according to expectation that the measurement of the step-like increase in capacitance of the patch at I_{on} (~ 0.22 pF)

³The simple apposition of the sperm and egg membranes, sufficiently close to exclude water and electrolytes from the region between them, would be expected to cause a slight decrease, rather than an increase, of the capacitance due to placing capacitors in series, or by increasing the thickness of the dielectric.

compares favorably with the estimate for the capacitance of the sperm head of 0.16 pF, much smaller than the capacitance estimated for the entire plasma membrane of the sperm.

Events that could increase membrane patch capacitance are exocytosis (Neher and Marty, 1982; Fernandez et al., 1984; Lindau and Fernandez, 1986; Breckenridge and Almers, 1987; Almers, 1990) of cortical granules (Jaffe et al., 1978; Kado, 1978; McCulloh, 1985) or a change of the egg's cortical/membrane tension (Hiramoto, 1963; Yoneda, Ikeda, and Washitani, 1978) leading to more egg plasma membrane being drawn into the patch pipette. Both of these events occur too late to account for the step-like increase in capacitance of the patch at the time of I_{on} . Electron microscopy has shown that dehiscence of the cortical granules in the vicinity of the attached sperm does not begin until 6–8 s after I_{on} (Longo et al., 1986). Even if, unexpectedly, a single cortical vesicle did fuse with the patch at the time of I_{on} , the increase in capacitance would be ~ 0.03 pF (surface area of a granule $\sim 3 \mu\text{m}^2$) or negligible for an individual cortical vesicle compared with the measured increase of ~ 0.22 pF for the patch.

Electrical continuity between the gametes once established at I_{on} is maintained when the sperm enters, but is disrupted when sperm entry fails. As noted previously, for eggs voltage clamped at potentials more negative than +20 mV, an abrupt increase of capacitance, indicative of the establishment of electrical continuity between the gametes, occurs simultaneously with I_{on} irrespective of whether or not sperm entry subsequently occurs. Only if the increase in capacitance is maintained, invariably associated with type I activation currents, does sperm entry occur. This is clearly according to expectation, since early during sperm entry the membranes of the gametes must fuse (Colwin and Colwin, 1967), resulting in cytoplasmic continuity and subsequent sperm incorporation. Conversely, sperm entry fails when the increased level of capacitance of the patch is not maintained, but rapidly decreases simultaneously with the cutoff of inward current at the termination of sperm transient currents (type II) or phase 1 of modified activation currents (type III). The rapid decrease of current after I_{on} signals that the sperm will not enter (Lynn et al., 1988). For sperm transient currents (type II), the rapid decreases of capacitance and current return these parameters to their prereshponse baselines. During the modified activation currents (type III), the rapid decrease of capacitance at the end of phase 1 did not return to its baseline value, since cortical granule exocytosis had already started several seconds before the end of phase 1. The rapid decrease of capacitance at the end of the sperm transient currents or phase 1 of modified activation currents signifies disruption of the electrical continuity between the gametes, which was first established at I_{on} . Observations supporting this conclusion are that the attached sperm which elicits a type II or III response floats or swims off the surface of the egg, or for type III responses is lifted off the surface by the rising fertilization envelope, conclusively causing loss of sperm–egg contact (Lynn et al., 1988). This frequently occurs within the first several seconds after the abrupt cutoff of current to the prereshponse baseline. It is not surprising that the sperm fails to enter the egg when electrical continuity between the gametes is not maintained. It is more surprising that the intimate electrical continuity between the sperm and egg, once established, can be disrupted.

Is the establishment of electrical continuity between the cytoplasm of the gametes due to fusion of the gametes' plasma membranes? The step-like increase in capacitance of the patch at I_{on} reveals the time when the sperm's membrane capacitance has become electrically accessed from within the egg's cytoplasm. This method, however, provides no information regarding how the electrical continuity between the gametes is achieved.

In electron microscope studies on inseminated *L. variegatus* eggs (the species used in this work) clamped at -20 mV, a potential at which sperm are readily incorporated by the eggs, fusion of the apposed plasma membranes of the gametes with formation of a connecting cytoplasmic bridge was not detected until 5 s after I_{on} , at the mid-interval of phase 1 (total duration ~ 12 s). The findings of Longo et al. (1986) are consistent with the results obtained in two other studies carried out using eggs inseminated in suspensions under conditions where the sperm readily enter the eggs. These are the studies on the transfer of a fluorescent probe from eggs of *L. variegatus* to attached sperm in specimens fixed at 1-s intervals after I_{on} (Hinkley et al., 1986), and the earlier electron microscope investigations on the gametes of other species (Colwin and Colwin, 1967).

The formation of a cytoplasmic bridge between the gametes at 5–8 s after I_{on} described by Longo et al. (1986) would be expected to provide electrical continuity between the gametes. However, in our patch clamp studies, electrical continuity occurred 5 s earlier, at I_{on} . No significant change in capacitance of the patch was detected near the earliest time corresponding to establishment of membrane continuity as seen with electron microscopy (between 5 and 8 s after I_{on}). (A second change in capacitance was detected only later, toward the end of phase 1, in association with the start of exocytosis of the cortical granules).

One possibility to explain these apparently conflicting findings is that fusion of the gamete plasma membranes had indeed occurred at I_{on} , but the just-fused membranes were in an unstable state for the first several seconds after I_{on} and consequently were not preserved during the fixation procedures used (Longo et al., 1986). Another possibility is that membrane fusion does not occur until mid-interval of phase 1, but this event is preceded by an intermediate, electrically conductive state of the apposed membranes. Longo, Cook, McCulloh, Ivonnet, and Chambers (1990) have presented evidence for the existence of an intermediate structure in bilayer fusion before the completion of fusion of the gamete membranes. This electron microscope evidence comprises a bimodal membrane profile indicative of fusion between the outer membrane leaflets of the sperm and the egg, similar to a fusion intermediate previously postulated by Palade and Bruns (1968) during fusion of secretory vesicles with the plasma membrane. The electrical properties of such a structure are not known, but might provide the electrical continuity observed in this paper. Alternatively, electrically conducting pores similar to gap junction channels, the only type of small pore known to traverse adjacent plasma membranes, have been proposed as an intermediate stage preceding fusion between the membranes of secretory vesicles and the plasma membrane (e.g., Almers, 1990). Channels of this type, ~ 20 Å in diameter (Berdan and Caveney, 1985; Veenstra and deHaan, 1988; Spray, Saez, Brosins, and Bennett, 1990) would not have been detected in the electron microscope studies of Longo et al. (1986), where by conservative estimate the lower limit of resolution

amounted to 50 A. The conductance of a single gap junction channel has been variously given as 45–240 pS (Berdan and Caveney, 1985; Spray et al., 1986; Veenstra and deHaan, 1988; Spray and Burt, 1990). Taking ~ 100 pS as the average unitary conductance, ~ 50 channels of the gap junction type, formed nearly simultaneously, could account for the conductance increase observed at I_{on} . We believe that this is unlikely.

Is Entry of the Sperm Precluded at a Step before or after Membrane Fusion When Nonpermissive Membrane Potentials Block Sperm Entry?

Eggs in which sperm entry is prevented by negative membrane potentials. Neither electron microscope nor dye transfer studies have been carried out to investigate the early stages of sperm–egg interaction in eggs where entry of sperm that attach to the egg is prevented by negative membrane potentials. Consequently, although electrical continuity is first established and then disrupted, the structural correlate of electrical continuity is not known for these eggs. Therefore, for eggs clamped at membrane potentials nonpermissive for sperm entry, we do not know whether continuity is lost by disruption of membrane fusion, the disruption of intercellular pores resembling gap junction channels, or a less characterized intermediate membrane structure (see above). During early stages of membrane fusion in exocytosis, capacitance occasionally fluctuates rapidly (Almers, 1990) between two discrete levels, suggesting instability of states or reversibility of the steps involved in establishing electrical continuity between the two membrane compartments. It is possible that such rapid fluctuations exist during sperm–egg fusion; but our recordings are carried out with insufficient bandwidth to resolve such rapid fluctuations. The rapid cutoff of capacitance at the termination of type II sperm transient currents and phase I of type III modified activation currents is our best evidence for instability during membrane fusion.

Sperm–egg fusion does not occur at positive membrane potentials that preclude sperm entry. The failure of sperm to enter eggs maintained at positive membrane potentials is evidence of an electrical block to polyspermy (Jaffe, 1976). When the membrane potential of eggs was maintained at values near +35 mV, sperm inside the pipette caused neither conductance nor capacitance increases. From this observation, we conclude that the fusion of sperm and egg membranes does not occur when the membrane potential of the egg is maintained at +35 mV. This is the first clear evidence that the block acts by precluding fusion of the sperm. The effect of positive egg membrane potentials to preclude membrane fusion must be mediated by a voltage sensor that can orient or move within the field of the egg's membrane. The work of Jaffe and co-workers (Jaffe, Gould-Somero, and Holland, 1982; Jaffe, Cross, and Picheral, 1983; Iwao and Jaffe, 1989) indicates that the voltage dependence of sperm entry at positive membrane potentials is ascribable to the sperm. Therefore, one plausible explanation is that the voltage sensor is a sperm molecule that is capable of inserting into the egg's membrane to sense the electric field.

Two distinct voltage sensors regulate establishment and maintenance of electrical continuity. It is tempting to view establishment and disruption of cytoplasmic continuity in eggs maintained at large, negative membrane potentials as proceeding forward and backward, respectively, between two discrete steps in a kinetic scheme with one energy barrier separating them. However, the existence of two distinct

voltage dependences for the forward and backward steps indicates that this tempting notion is not correct. Electrical continuity is established only when the egg's membrane potential is more negative than +20 mV. Sustained electrical continuity is assured only if the egg's membrane potential is maintained more positive than -20 mV. Therefore, sperm entry (requiring both establishment and maintenance of electrical continuity) exhibits two very different voltage dependences with distinct ranges of voltage dependence and with opposite polarities. This requires two distinct voltage-sensing mechanisms. The voltage dependences must occur at two distinct kinetic steps. Therefore, establishment of electrical continuity and disruption of continuity occur by two distinct kinetic transitions and not by reversal of the same step.

Abrupt Changes in the Capacitance and Conductance of the Patch Are Obligatory Coupled

The abrupt increases of both the capacitance and conductance of the patch at the time of I_{on} for all the responses elicited by the sperm occurred simultaneously, as did the rapid decreases of capacitance and conductance of the patch at the termination of the sperm transient currents (type II) and phase 1 of modified activation currents (type III). In addition, at potentials more positive than +20 mV, the sperm did not elicit any change in either the capacitance of the patch or the membrane conductance. These coincidences indicate that changes in capacitance of the patch and conductance are obligatorily associated (Table V).

A straightforward explanation for the coupling of these events at I_{on} is that the membrane surface area responsible for the increase in capacitance of the patch, and the membrane channels responsible for the increase in conductance of the patch, are both localized to the sperm's membrane. Consequently, when electrical continuity between the gametes' cytoplasm is established at I_{on} , the sperm's membrane capacitance is added to that of the patch, necessarily at the same time that the sperm's membrane channels are introduced. Conversely, when electrical continuity between the gametes is disrupted, both the membrane capacitance and the membrane channels of the sperm are simultaneously no longer accessed. Consistent with this explanation is our finding in the patch clamp studies that the increase in the whole egg's conductance during phase 1 of type I and III responses, and the type II sperm transient currents are localized to the site, or the immediate vicinity, of the attached sperm. On the basis of these findings, in the voltage clamped egg during phase 1, the conductance of essentially the entirety of the egg's plasma membrane ($4 \times 10^4 \mu\text{m}^2$) remains at the exceedingly low level of ~ 2 nS characteristic for the unfertilized egg. The increase in conductance at I_{on} to ~ 7 nS is achieved by insertion of a 5-nS conductance in parallel with the egg's membrane conductance. If the entirety of the conductance increase at I_{on} is due to channels introduced by the sperm ($\sim 86 \mu\text{m}^2$ surface area) at the time electrical continuity is achieved, as we propose, ~ 50 channels with a unitary conductance of 100 pS would suffice. This amounts to ~ 0.5 channels/ μm^2 . That this density of channels could exist in the sperm's plasma membrane is not unlikely (Hille, 1984). Single channel currents have been demonstrated in sperm before the acrosome reaction (Guerrero, Sanchez, and Darszon, 1987). The sperm that binds to the egg must have already undergone the acrosome

reaction (Dan, 1956), after which additional channels are presumed to open, since the sperm's membrane permeability increases dramatically and the membrane depolarizes toward 0 mV (Schackman, Christen, and Shapiro, 1981, 1984; Garcia-Soto, Gonzalez-Martinez, DeLaTorre, and Darszon, 1987). On the basis of the proposal advanced here, the channels introduced in the sperm's membrane should primarily determine the characteristics of the phase 1 currents. This is in accordance with the observation that the rough estimate of 0 mV for the membrane potential of the acrosome reacted sperm is not particularly divergent from the reversal potential for the current generated at I_{sm} (end of phase 1), approximately +17 mV (Lynn et al., 1988). Moreover, in cross-fertilization experiments on voltage clamped echinoderm eggs, Kane (1990) has shown that the characteristics of the currents generated as early as 10 s after sperm attachment are determined by the species of the sperm used, rather than by the species of the egg. This contrasts with membrane potential records from homologous and cross-species fertilizations of the amphibians *Cynops* and *Hynobius* (Iwao, 1989; Iwao and Jaffe, 1989).

A Second, Smooth Increase of the Capacitance Indicates the Time of Initiation of the Exocytosis of Cortical Granules

The onset of a second increase of capacitance beginning 8 s after I_{on} occurs before deterioration of the seal resistance, corresponding to the time when cortical granules begin to dehisce (Longo et al., 1986) in the neighborhood of the attached sperm. The exocytosis of cortical granules is associated with an increase of the egg's membrane capacitance (Jaffe et al., 1978; Kado, 1978; McCulloh, 1985) and is observed earlier for the patch near the sperm than was detected previously using whole egg techniques.

Cortical granules are smaller than sperm. Consequently, the expected capacitance increase caused by the fusion of one cortical granule would be difficult to detect given the noise level seen in our records. However, when several cortical granules fuse in succession, a smooth increase of capacitance results (Jaffe et al., 1978; Kado, 1978; McCulloh, 1985). The second increase of capacitance occurred only in eggs that underwent exocytosis as indicated by elevation of the fertilization envelope (either type I activation currents or type III modified activation currents) but was absent in eggs where sperm evoked only type II sperm transient currents and no elevation of the fertilization envelope occurred (Table VI). We conclude that the second increase of capacitance is indicative of the initiation of the exocytosis of cortical granules in the region of the egg monitored by the loose patch clamp pipette, near the site of the attached sperm.

Is Establishment of Electrical Continuity Required for Activation?

The two types of electrophysiological responses for which sperm entry fails indicate that the egg can either be activated (type III, modified activation current) or may remain dormant (type II, sperm transient current) during and after its interaction with the sperm. Electrical continuity between the sperm and the egg was established in both cases and yet the egg was activated only during the modified activation current. However, activation of an egg, when it does occur, is always preceded by the establishment of electrical continuity between the cytoplasms of the sperm and the

egg (type I and III responses). We conclude that establishment of electrical continuity always preceded egg activation. Although this suggests that establishment of electrical continuity is required for activation, electrical continuity is not sufficient. Whether the insufficiency of electrical continuity is due to differences in the duration over which the continuity is maintained or differences of the quality or properties of the structure that establishes the continuity, or whether another mechanism such as an extracellular receptor-ligand interaction (Gould and Stephano, 1987; Jaffe, 1990) is also required cannot be concluded from the data presented in this paper. However, it is important to note that electrical continuity is established ~ 8 s before any outward signs of the onset of activation in the egg, thereby proving that a form of cytoplasmic continuity is established early enough to permit the flow of signals directly between the cytoplasms of the egg and the sperm (Dale, DeFelice, and Ehrenstein, 1985; Whitaker, Swann, and Crossley, 1989). Therefore, in considering the mechanism by which the sperm activates the egg, the possibility of cytoplasmic signals must be considered.

CONCLUSION

Several years ago, Jaffe and co-workers (Gould-Somero and Jaffe, 1984; Jaffe and Cross, 1986) concluded that the positive membrane potential attained in the first seconds after a successful sperm attachment serves to block the fusion of supernumerary sperm. This concept for a rapid block to polyspermy (Jaffe, 1976) was developed at a time when it was believed that sperm-egg fusion occurred near the end of the latent period (Epel et al. 1977; Whitaker and Steinhardt, 1985). If this were the case, how could the first sperm fuse? The evidence presented in this paper suggests that sperm-egg membrane fusion occurs or is at least initiated at the time of I_{on} . Since establishment of electrical continuity coincides with I_{on} , it too is the earliest physiological sign of successful contact between sperm and egg. As the first event, the establishment of electrical continuity assures that fusion is initiated before attainment of the positive membrane potential that precludes fusion. A fail-safe mechanism to achieve this is provided if the channels responsible for initiating the depolarization reside in the sperm's membrane and become available to the egg's membrane only after the establishment of electrical continuity. Therefore, the fertilizing sperm is responsible for the rapid block to polyspermy, not only because the sperm's membrane is the likely site of the voltage sensor (Jaffe et al., 1982), which responds to the imposed positive potential, but also because the channels that initiate the depolarization of the egg's membrane potential reside in the sperm's membrane. In addition to the sperm's role in providing a fail-safe mechanism to block polyspermy, the depolarization induced by introduction of the sperm's membrane channels provides the depolarization necessary to permit that sperm's entry (Lynn et al., 1988). Therefore, the establishment of electrical continuity by the first sperm assures that no other sperm will fuse with the egg, and that the first sperm to fuse will enter the egg.

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