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Calcium Influx Mediates the Voltage-Dependence of Sperm Entry into Sea Urchin Eggs

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Sperm entry was monitored in voltage-clamped sea urchin eggs following insemination in a variety of artificial seawaters. In regular seawater, maintaining the membrane potential at increasingly negative values progressively inhibits sperm entry. Reducing $[Ca^{2+}]_o$ relieves the inhibition, shifting the sperm entry vs voltage relationship toward more negative potentials. Raising $[Ca^{2+}]_o$ shifts the relationship in the other direction. Large changes in $[Na^+]_o$ or $[Mg^{2+}]_o$ do not affect sperm entry although changing $[Na^+]_o$ dramatically changes the currents following sperm attachment. Applying one of seven different calcium channel blockers or replacing Ca^{2+} with Ba^{2+} or Sr^{2+} or microinjecting calcium chelators into the cytoplasm relieves the block to sperm entry at negative potentials. We conclude that the block to sperm entry at negative potentials is mediated by calcium which crosses the membrane and acts at an intracellular site.

INTRODUCTION

Sperm–egg interactions are regulated by membrane potential (V_m)[2] in sea urchins and in several other species (Jaffe, 1976; Jaffe *et al.*, 1983; Cross and Elinson, 1980; Lynn and Chambers, 1984). Maintaining the egg's V_m more positive than +5 mV or more negative than −20 mV decreases sperm entry in sea urchin eggs. The nature of the inhibition at negative potentials is entirely different from that at positive potentials. When failure of sperm entry occurs at positive potentials, the egg displays neither an electrophysiological response (EPR) nor any other sign of activation despite the sperm's attachment to the surface of the egg (Jaffe, 1976; Lynn and Chambers, 1984).

The block of the egg's response to the sperm is achieved by preventing fusion at positive V_m (McCulloh and Chambers, 1992), which serves as a rapid block to polyspermy (Jaffe, 1976) and will not be discussed further here, except incidentally.

When sperm entry is blocked in sea urchin eggs by maintaining the egg at negative potentials the attached sperm elicits an EPR (Lynn and Chambers, 1984; Lynn *et al.*, 1988). The block occurs after electrical continuity between the aqueous phases of the two gametes' cytoplasm is established coincident with initiation of an EPR at I_{on} (McCulloh and Chambers, 1992). The impending failure of entry is first signaled by loss of electrical continuity coincident with sudden diminution of the EPR at I_{off} (Lynn *et al.*, 1988; McCulloh and Chambers, 1992).

The object of this research was to study the mechanism whereby negative voltage exerts its regulatory effect on sperm entry. Calcium was identified as a mediator of the voltage dependence via calcium influx and local intracellular accumulation in the region near the sperm attachment site.

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[2] Abbreviations used: V_m, membrane potential; EPR, electrophysiological response; I_{on}, the initial onset of current; I_{sm}, the maximum current of the shoulder (phase 1) of the activation currents of eggs penetrated by sperm; I_p, the major current peak of activation currents; I_{off}, the abrupt cessation of transient currents associated with the failure of sperm entry into eggs voltage-clamped at negative potentials; BAPTA, 1,2-bis(2-aminophenoxy)ethane tetraacetate; Pipes, 1,4-piperazinediethane sulfonate; TAPS, *n*-tris(hydroxymethyl)methyl-3-aminopropane sulfate; DMSO, dimethyl sulfoxide.

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BACKGROUND

The earliest events observed following attachment of the sperm to the egg are coincident establishment of electrical continuity between the gametes' cytoplasms and an initiation of an EPR at I_{on}. In unclamped eggs the EPR comprises the onset of inward current that results in an approximately 100-mV depolarization of the egg's V_m from a resting value of −70 to −80 mV to about +30 mV (Chambers and deArmendi, 1979). The sustained positive-going potential occurs in two phases. Phase 1 is initiated by an initial local membrane conductance increase near the sperm, which depolarizes the egg and, in about 100 ms, triggers a global Ca^{2+}-dependent action potential. After partial repolarization to the neighborhood of +10 mV, lasting about 12 s, Phase 2, a positive-going potential, ensues, rising to about +25 mV by 30 s, following which the potential slowly returns to a negative value near that of the unfertilized egg. Phase 2 results primarily from an increase in cation conductance that sweeps over the surface of the egg as a band from the sperm's site of attachment to the antipode. The surface wave is associated with a wave-like elevation of cytoplasmic free calcium levels (Swann *et al.*, 1992) that propagates throughout the cytoplasm of the egg (McCulloh and Chambers, 1991).

If, during insemination, the egg is not permitted to depolarize from its resting potential then sperm that had established cytoplasmic continuity fail to enter the egg. Sperm that fail to enter often detach from the egg and either fall to the bottom of the dish or are lifted off the surface of the egg by the rising fertilization envelope. To explore this phenomenon more fully, eggs were voltage clamped and both the ability of the sperm to elicit an EPR and the type of EPR were recorded. In normal seawater, sperm attach at all potentials examined (+50 to −120 mV) and elicit EPRs at all V_ms more negative than +18 mV (Lynn *et al.*, 1984, 1988). Between +5 and +18 mV, many sperm may attach before an EPR is elicited, while at potentials more negative than +5 mV, the great majority of sperm that attach elicit an EPR (Lynn *et al.*, 1988).

Initiation of an EPR Is Associated with Sperm–Egg Fusion

An EPR signifies that electrical continuity between the cytoplasms of the sperm and egg has occurred (McCulloh and Chambers, 1992). Sudden establishment of electrical continuity results in the abrupt onset of inward current, I_{on}, localized to the region of the egg near the sperm. Establishment of electrical continuity between the sperm and the egg may be achieved by fusion of sperm and egg membranes (McCulloh and Chambers, 1992). However, continuity of the membrane constituents has been documented by fixation and electron microscopy only 4–5 s after initiation of an EPR (Longo *et al.*, 1986, 1994). Therefore, we use the conservative terms "establishment of electrical continuity" or "establishment of cytoplasmic continuity" rather than "sperm–egg fusion" to refer to this event whereby direct communication between the cytoplasms of the two gametes first occurs.

Type I EPR Is Associated with Entry of a Sperm and Egg Activation

Elicitation of an EPR by a sperm in eggs clamped at −10 to +18 mV is invariably associated with sperm entry (see Fig. 1), followed by syngamy (union of the male and female pronuclei), and then by mitosis and cell division. The EPR elicited by an entering sperm has a characteristic pattern and is termed the activation current (Type I EPR; Lynn *et al.*, 1988; Chambers, 1989). The inward current following I_{on} steadily increases at first slowly (Phase 1), then rapidly (Phase 2), to attain a peak (I_p) at about 30 s (see Fig. 2, upper trace).

Type II and Type III EPRs and Abrupt Loss of Cytoplasmic Continuity Are Associated with Failure of Sperm Entry (with or without Egg Activation)

At increasingly negative V_ms from −10 mV, the proportion of sperm that fail to enter despite causing an EPR increases until at −80 mV (resting V_m of unfertilized egg), sperm entry is completely inhibited (see Fig. 1; Lynn and Chambers, 1986; Lynn *et al.*, 1988). The failure of sperm entry is invariably signaled by two coincident events, loss of cytoplasmic continuity (McCulloh and Chambers, 1992) and a rapid cutoff of the current, I_{off}. These events occur anywhere from 1 to 20 s (average 12) after the initial abrupt onset of inward current, I_{on}. The sperm either remains immotile at the egg's surface or drifts away. If after I_{off} the current returns to and remains at the preinsemination level, the EPR is termed a sperm transient current (Type II EPR), and the egg remains in the unfertilized and inactivated state. Sperm transient currents are either of long duration (over 3.0 s, mean of 10.6 s) or of short duration (less than 3.0 s, mean of 1.7 s).

Alternatively, after the loss of cytoplasmic continuity and I_{off}, the inward current increases at first slowly and then more rapidly to attain a delayed peak. This type of EPR is termed a modified activation current (Type III EPR). The fertilization envelope elevates fully or partially, but no cell cleavage occurs in association with the absence of a sperm centriole or aster.

At increasingly negative potentials between −20 and −70 mV, for which sperm entry is increasingly inhibited, activation currents are increasingly replaced by modified activation currents and sperm transient currents of long duration in approximately equal numbers. More negative than −70 mV, which fully suppresses sperm entry, long-duration sperm transient currents increasingly replace the modified activation currents, and more negative than −80 mV,

short-duration sperm transient currents increasingly replace long duration sperm transient currents.

Purpose of These Experiments

Sperm entry is dependent upon the membrane potential of the egg. A membrane-potential-dependent event means there must be an ion or dipole that resides, at least temporarily, within the membrane's potential field and that responds to the membrane's electrical field. The charge movement associated with the voltage dependence for sperm entry (*e*-fold change of sperm entry for 7.6 mV) was estimated to be equivalent to a minimum of three or four electron charges displaced across the total electrical field (McCulloh, 1989). In the experiments described here, we wanted to characterize the agent that responds to the membrane's electrical field. Ion substitution experiments were performed, as ions are likely candidates for mediators of voltage dependence that are exposed to the membrane field during transmembrane flux. Alternatively, ion substitution may affect intramembrane voltage sensors because extracellular ions may also influence the membrane field either locally or more globally by adsorption to the membrane surface and alteration of the membrane's surface charge.

MATERIALS AND METHODS

Gametes

Eggs of the sea urchin *Lytechinus variegatus* were obtained and jelly-free unfertilized eggs prepared as described by Lynn and Chambers (1984) and McCulloh and Chambers (1991). Experiments were carried out at 22–23°C.

Electrophysiological Recordings

Whole eggs were voltage clamped using a switched single-electrode voltage clamp (Model 8100 or 8800; Dagan Corp., Minneapolis, MN; Wilson and Goldner, 1975), equipped with a bridge circuit in order that the V_m of the egg could be determined both before and after measurements made in the voltage clamp mode. A pulse generator (Model 184; Wavetek, San Diego, CA) was used to apply test pulses to the unclamped egg or voltage command pulses to the voltage-clamped egg. Current and voltage were displayed on either a three-channel storage display oscilloscope (Model 5100 series; Tektronix, Inc., Beaverton, OR) or a digital oscilloscope (Model 3091; Nicolet Instrument Corp., Madison, WI).

An additional oscilloscope (Model V212; Hitachi Denoshi, Ltd., Tokyo, Japan) was used to continuously monitor the headstage voltage of the switched single-electrode clamp at high time resolution during voltage clamping. The current and voltage were recorded on a two- or four-channel ink recorder (Model 2200 or 2400 S; Gould Electronics) and also on a four-channel tape recorder (either Model 3964A; Hewlett Packard, Corvallis, OR; or Model Store 4 DS; Racal Recorders Ltd., Hardley Industrial Estate, Southampton, England). Tape recording permitted vocal description of procedure as the experiment progressed, as well as later playback of the currents and voltage data at the desired amplification and time interval.

Electrodes

When electrophysiological measurements were to be carried out and compared for eggs suspended in the bath perfused with different ionic media, the bath electrode used was a Ag–AgCl pellet–3 M KCl–2% agar bridge in 3 M KCl. The 3 M KCl served to minimize the junction potential where the tip of the bath electrode made contact with the bath. When all the measurements were to be made on eggs in seawater (SW), the bath electrode contained SW instead of 3 M KCl. The bath electrode was connected to the instrument ground of the switched single-electrode clamp.

Microelectrodes were pulled using thin-walled borosilicate glass tubing 1 mm o.d. × 0.75 mm i.d. containing a fine fiber (No. 30-30-0; Frederick Haer, Brunswick, ME). The external surface of each microelectrode was painted for a distance of ~1 cm starting 20–40 μm from the tip with polystyrene paint (Q dope; GC Electronics, Rockford, IL). This insulation step alone reduced the time constant of the microelectrode to values which permitted effective voltage clamping of the egg.

The microelectrodes were filled with a solution containing 0.5 M K_2SO_4, 20 mM NaCl, and 0.5 mM K citrate. Those selected for voltage clamping had a resistance of 20–30 MΩ. The filled microelectrodes were connected into the circuit using a Lucite microelectrode holder with side port and Ag wire (No. EH-2MSW; E. W. Wright, Guilford, CT). After capacitance compensation, current-induced changes of microelectrode potential settled in <70 μS.

Solutions

The composition of artificial SW and the ion-substituted SW mixtures used are listed in Table 1.

The artificial SW was prepared according to the formula of Lyman and Fleming (1940), but with salinity increased to 36%, that of Gulf Stream SW at Miami, Florida. The trace ions Br^-, F^-, BO_3^{3-}, and Sr^{2+} were omitted. Bicarbonate (HCO_3^-) was replaced with either 10 mM TAPS or glycylglycine as a buffer and adjusted to pH 8.3. The osmolarity of each of the SW mixtures was checked and adjusted by adding NaCl to be the same as that of the Gulf Stream SW.

The composition of the 1.5 mM Na^+ SW mixture was identical to artificial SW (Table 1) except that choline replaced the removed Na. The Ba^{2+} and Sr^{2+} SW mixtures were prepared in SO_4^{2-}-free SW (Table 1) with 10.7 mM Ba^{2+} or Sr^{2+} replacing the Ca^{2+}.

Changes in Mg^{2+} were accompanied by osmotically equivalent changes in Na^+. Ca^{2+} was decreased below the 10.7 mM of regular SW by isosmotic substitution of $CaCl_2$ with $MgCl_2$. Increases of Ca^{2+} were accomplished by isosmotic substitution of $CaCl_2$ for NaCl while Mg^{2+} was maintained constant. The concentrations of Ca^{2+} given in Table 1 were measured by atomic absorption spectrophotometry because we were concerned about the amount of water in the hygroscopic solid $CaCl_2$.

The stock solutions of the Ca^{2+} channel blocking agents (verapamil, diltiazem, nifedipine, and nimodipine) were prepared in DMSO and added to SW at the designated concentrations in such a way that the concentration of DMSO did not exceed 0.125%. This concentration of DMSO had no detectable effect on sperm entry and development of the eggs.

TABLE 1
Compositions of the Solutions

	$[Na^+]_o$	$[K^+]_o$	$[Ca^{2+}]_o$	$[Mg^{2+}]_o$	$[Cl^-]_o$	$[SO_4^{2-}]_o$	[Buffer][a]
Artificial SW	493	10.4	10.7	54	570	29	10 (T)
260 mM Mg^{2+} SW	194	10.4	10.7	260	683	29	10 (T)
10.8 mM Mg^{2+} SW	558	10.4	10.7	10.8	548	29	10 (T)
1.0 mM Ca^{2+} SW	493	10.4	1.0	63.7	570	29	10 (T)
3.2 mM Ca^{2+} SW	493	10.4	3.2	61.5	570	29	10 (T)
22.1 mM Ca^{2+} SW	476	10.4	22.1	54	576	29	10 (GG)
69.3 mM Ca^{2+} SW	406	10.4	69.3	54	600	29	10 (GG)
89 mM Ca^{2+} SW	376	10.4	89	54	610	29	10 (GG)
SO_4^{2-}-free SW	478	10.4	10.7	54	613	0	10 (T)

[a] T, TAPS; GG, glycylglycine.

Source of Reagents

All organic chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). The inorganic chemicals were reagent grade (Baker Analyzed).

General Experimental Procedure

For experiments in which the ionic solution bathing the eggs was exchanged, a perfusion chamber was used. This consisted of the lid of a petri dish, 60 × 10 mm (Falcon 3002; Becton–Dickinson and Co., Lincoln Park, NJ), containing a polymerized insert consisting of Sylgard (Dow-Corning, Midland, MI) into which a trough had been carved, measuring 40 mm in length, 15 mm in width at the center, 5 mm in width at each end, with a thickness of ~3.5 mm. The trough had a capacity of ~3 ml with an inlet and an outlet at each end. Solution flowed into the trough by gravity and was sucked out by gentle aspiration using a water aspirator, maintaining the SW at a constant depth of ~2.5 mm. The perfusion chamber was mounted on the mechanical stage of an inverted microscope (Model IM-35; Zeiss, Oberkochen, Germany). Two milliliters of SW and then from 50 to 100 jelly-free eggs were pipetted into the trough of the perfusion chamber.

Eggs on the floor of the chamber were observed at 400× magnification using either a Zeiss 40× (0.60 NA) long working distance (1.5 mm) Planachromat objective or a Zeiss 40× (0.65 NA) Planachromat objective with a working distance of 0.7 mm. Eggs suitable for insertion of a microelectrode were those that loosely adhered to the floor of the perfusion chamber. In all experiments the microelectrode was inserted into the egg while immersed in regular SW. Insertion of the electrode into the egg, sealing, measurements of input resistance, and procedure for voltage clamping were as described in Chambers and deArmendi (1979), Lynn and Chambers (1984), and McCulloh and Chambers (1991).

After satisfactory sealing of the egg in SW had been attained and the V_m stabilized, the egg was tested for its ability to generate an action potential (Chambers and de Armendi, 1979) following application of a depolarizing current pulse (from a V_m of approximately −70 mV). In many eggs the resting V_m approximated −20 mV. For these eggs, the ability to generate an action potential was tested first by application of hyperpolarizing current to maintain the V_m near −70 mV and then sudden cessation of the hyperpolarizing current. Voltage clamp was then applied, clamping the V_m of the egg at its resting potential (the 0 current level). The input resistance of the egg was measured with the egg clamped at −20 mV, and also at −70 mV, by application of 10 mV hyperpolarizing command pulses (duration ~0.5 s). Eggs lacking an action potential, or having an input resistance less than 100 MΩ at −20 mV, were discarded.

Solution changes. After electrode insertion and sealing, the medium surrounding the egg could be changed. To change the medium, 30–40 ml of solution was perfused through the chamber. This represents a 10-fold exchange of the medium originally present in the chamber. After completion of the perfusion, the input resistance of the egg was again measured at −20 and at −70 mV.

Insemination. To inseminate (Lynn and Chambers, 1984), 2 μl solid (or "dry") sperm was diluted in 40 ml of regular SW or of the ion-substituted SW mixture in which the egg was suspended. Then 100 μl of the diluted sperm was gently pipetted into the bath (2–3 ml volume) in the vicinity of the egg with the microelectrode inserted.

For the ion-substituted SW mixture containing 1 mM Ca^{2+} the sperm had first to be treated to induce the acrosome reaction (Takahashi and Sugiyama, 1973) in order to achieve fertilization. To do this 20 μl of solid sperm was diluted in 4 ml of SW containing egg jelly. After being stirred for 20 s, 0.5 ml of the treated sperm (having been caused to undergo the acrosome reaction) was made up to 5 ml with Ca^{2+}-free SW. One hundred microliters was removed and then added to the bath containing the eggs within 10 s. Treated sperm were also used to inseminate eggs in low Na^+, Ba^{2+}, and Sr^{2+} SW mixtures.

Microinjection of EGTA or BAPTA

The chelating agents, EGTA and BAPTA, were each prepared from their potassium salts at concentrations from 10 to 500 mM in 0.35 M K_2SO_4 with 10 mM Pipes buffer, pH 6.9. Microinjections were performed with one of these solutions in the injection pipette essentially as described by Swann and Whitaker (1986). Brief pulses of pressure were applied to the interior of the injection pipette roughly once per second. As the procedure was videotaped, the pipette was inserted into the egg. Access to the cytoplasm was verified by the appearance of a rapidly disappearing spherical bolus with each pressure pulse. After the experiment, the videotaped

procedure was reviewed and the initial diameter of the first or second injected bolus (d_{bolus}) was measured to estimate its volume. The diameter of the egg (d_{egg}) was measured. The number of injected boluses (n) was counted. The concentration of EGTA or BAPTA was estimated from the concentration of chelator in the pipette ([chelator]$_p$) according to the equation

$$[\text{chelator}]_{egg} = n \times [\text{chelator}]_p \, (d_{bolus}/d_{egg})^3.$$

Quantitative Estimation of the Percentage of Sperm That Enter

As long as sperm suspensions are freshly prepared in normal SW, nearly every sperm that attaches to an egg elicits an electrophysiological response for eggs clamped at V_m more negative than +5 mV (Lynn *et al.*, 1988). However, when eggs are suspended in ionic media different from SW, the number of attached sperm which elicit an EPR varies widely. Consequently, in this paper we neglect sperm attachments that fail to elicit an EPR.

Much of our data was interpreted within the framework (Chambers and McCulloh, 1990)

Attachment of sperm to egg surface → Establishment of cytoplasmic continuity (Phase 1) → Incorporation of sperm

in which sperm attachment precedes establishment of cytoplasmic continuity between the sperm and the egg. The electrophysiological response (I_{on}) initiates coincident with establishment of cytoplasmic continuity (McCulloh and Chambers, 1992), which is a prerequisite for sperm-induced egg activation and for sperm entry. Sperm entry is not required for activation. The focus of this paper is the transition between establishment of cytoplasmic continuity and sperm entry. We previously demonstrated that this is regulated by the egg's membrane potential. In order to study this process in isolation, we restrict ourselves to consideration of only those sperm–egg interactions that achieve the establishment of cytoplasmic continuity, as indicated by the occurrence of I_{on}. When eggs are suspended in ionic media different from SW, the number of attached sperm that elicit an EPR varies widely. Sperm that attach but do not elicit an I_{on} are neglected in this paper. The entry of sperm into eggs measured electrophysiologically as the occurrence of a Type I EPR, under different experimental conditions, is expressed as the percentage (%) of the total number of EPRs elicited by attached sperm. The validity of expressing sperm entry in this way is that: (1) unless a sperm that attaches to an egg elicits an EPR, the egg remains completely unaffected, i.e., there is no evidence that even the earliest stage of gamete interaction is initiated (other than attachment of the sperm to the egg); (2) an egg is never penetrated by a sperm unless it elicits an EPR; and (3) one sperm can elicit only a single EPR.

When an egg displays only a single EPR (monospermic egg), whether the sperm entered can be readily determined from the type of EPR (Types I, II, or III) elicited (see Background). Only a Type I EPR signifies that sperm entry has occurred:

$$\%\ \text{sperm entry} = 100 \times \text{No. Type I EPRs/Total No. EPRs.}$$

For monospermic eggs (most of the sperm entries in this paper), verification is obtained by observing whether a sperm aster appears followed by cleavage in eggs surrounded by a fertilization envelope.

TABLE 2

The Association between Sperm Entry and Electrophysiological Response

	Sperm entry (aster seen)	No sperm entry
Type I—activation current—I_{on} without a following I_{off}	160	2
Type II or III—sperm transient current or modified activation current—I_{on} followed by I_{off}	0	308

Note. Each sperm entry was scored by the appearance of a sperm aster. 60 cases of Type I sperm entry and 191 cases of Type II or III nonentry from McCulloh (1989) are included.

In constructing the expanded plot of entry vs membrane potential (Fig. 1, under Results), it was necessary to determine whether sperm entered when several EPRs superimposed. When several sperm attach, they may elicit several EPRs in a single egg. This happens frequently in voltage-clamped eggs, since the positive shift of membrane potential (the fast block to polyspermy; Jaffe, 1976), which is normal in unclamped eggs, cannot occur. Even when the responses superimpose, the number of sperm that enter the egg can be estimated by counting the number of I_{on}s, and the number of sperm that fail to enter the egg is estimated by counting the number of I_{off}s, the characteristic abrupt cutoff of inward current that signifies disjunction of a sperm from the egg (see Background). In essence, when several EPRs superimpose (polyspermic eggs),

$$\%\ \text{sperm entry} = 100 \times (\text{No. } I_{on}\text{s} - \text{No. } I_{off}\text{s})/\text{No. } I_{on}\text{s}.$$

When EPRs were diminished in amplitude (in ion substitution and calcium channel blocker experiments), I_{on} and I_{off} were less obvious. In these experiments, the entry of sperm was confirmed by the appearance of sperm asters and, for monospermic eggs, cleavage of the egg.

RESULTS

Tight Association between Type EPR and Sperm Entry

The sperm's entry or failure to enter in SW is indicated by the type of EPR (Table 2). Nearly every sperm that evoked a Type I EPR entered its egg as confirmed by formation of a sperm aster. When one sperm entered, normal cleavage ensued within 1 h. In some cases, additional sperm contributed to Type I EPRs as additional I_{on}s during the Type I EPR. If no I_{off} was seen, the sperm was included as having evoked a Type I EPR and an additional aster was seen, except in two cases (addressed below). If an I_{off} was observed following an additional I_{on} during a Type I EPR, one EPR was scored as a Type I EPR and another was considered a Type II EPR. Each sperm that evoked a Type II or Type III response failed to

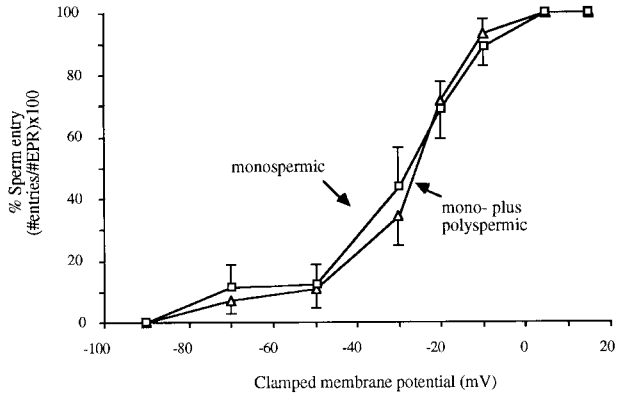

FIG. 1. The percentage of sperm entry in eggs clamped at different V_ms is the same when the only eggs counted are those penetrated by a single sperm (squares) or when eggs penetrated by multiple sperm are included (triangles). (Two-thirds of the sperm entries were in polyspermic eggs.) Error bars, ±SE. Sperm entry was confirmed by observing sperm asters. Five hundred six EPRs were recorded including 240 from Lynn and Chambers (1983) and Lynn *et al.* (1988).

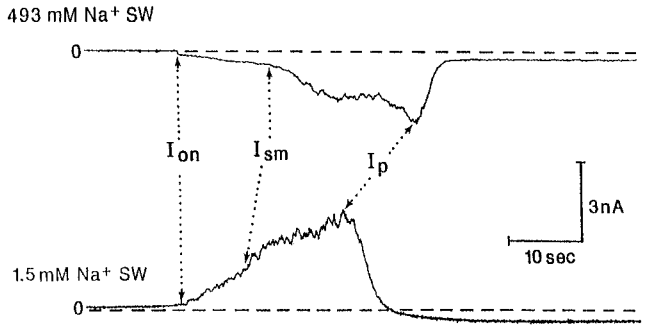

FIG. 2. Activation current in low-sodium seawater. Both eggs were clamped at −20 mV.

enter (no aster appeared). Asters and cleavage were never seen in eggs that underwent exclusively Type II or Type III EPRs. Cleavage was seen only if a sperm evoked a Type I response in that egg. A significant association exists between sperm entry and the EPR type. A G test of independence (with Yate's correction: $G = 569.6$ with 1 degree of freedom; $P \ll 0.005$) (Sokal and Rohlf, 1973) indicated that the observed incidence of entry was strongly associated with the type of EPR. We speculate that the failure to observe asters in 1% of the eggs showing Type I responses was a consequence of injury.

Effect of Clamped V_m on Sperm Entry for Eggs in SW

The inhibitory effect on sperm entry of voltage clamping eggs suspended in regular SW at negative V_m is shown in Fig. 1, which includes previously reported experiments (Lynn and Chambers, 1984; Lynn *et al.* 1988). Sperm were added in concentrations so low that usually only one would interact with the egg. The experiments with monospermic eggs are represented by the squares.

Occasionally, more than one sperm would attach to the surface of the egg and evoke an EPR. We now increase the data set by including the data for eggs with more than one EPR, some of which were temporally superimposed. Figure 1 (triangles) is a plot in which percentage sperm entry is calculated by taking the total of all EPRs elicited, irrespective of whether only one EPR had been elicited by a single sperm or several EPRs by multiple sperm in single eggs (monospermic plus polyspermic responses = total responses). The two plots are the same, within counting error. This validates the method of scoring sperm entry for polyspermic eggs in SW by counting the No. I_{on}s − No. I_{off}s.

Computing sperm entry from total responses has the advantage that each data point is based on a far larger number of EPRs, which reduced the standard error. In all the work reported in this paper, percentage sperm entry is computed on the basis of total number of responses elicited. (It should be pointed out that in Fig. 1, even though measurements were carried out at V_ms more positive than +18 mV, no data points are shown. This is because at such positive V_ms, attached sperm fail to elicit EPRs and, of course, to enter the egg. Consequently, % sperm entry = 0/0, an undefined number.)

Effects of Altering $[Na^+]_o$, $[Mg^{2+}]_o$, or $[Ca^{2+}]_o$ on Sperm Entry

Chambers and McCulloh (1990) observed that the amplitude of inward currents during Phase 1 of all EPRs increased with increasingly negative V_ms over the range in which sperm entry was increasingly inhibited. Cations are the predominant charge carriers during Phase 1 and influx of cations precedes the earliest indication of sperm entry failure (loss of cytoplasmic continuity at I_{off}). Therefore, the influx of cations was investigated as a candidate for media-

TABLE 3

Decreasing $[Na^+]_o$ 330-fold Does Not Affect Percentage Sperm Entry

	Control	Low $[Na^+]_o$
$[Na^+]_o$ concentration	493 mM	1.5 mM
% Sperm entry, mean ± SE (n)	9.1 ± 5.9% (22)	13.6 ± 7.3% (22)

Note. Eggs were voltage clamped at −70 mV. Sperm entry was confirmed by observing sperm asters.

TABLE 4

A Fivefold Increase or Decrease of $[Mg^{2+}]_o$ Does Not Affect Percentage Sperm Entry

	High $[Mg^{2+}]_o$		Low $[Mg^{2+}]_o$	
	Control, 54 mM	260 mM	Control, 54 mM	10.8 mM
% Sperm entry, mean ± SE (*n*)	44.0 ± 9.9% (27)	44.4 ± 10.4% (23)	34.8 ± 9.9% (32)	37.5 ± 8.6% (27)

Note. Eggs were voltage clamped at −30 mV. Sperm entry was confirmed by observing sperm asters.

tor of the inhibitory effect of V_m on sperm entry. We altered the concentration of the principal cations in SW (Na^+, Mg^{2+}, or Ca^{2+}) to see if they would modify the voltage dependence of sperm entry.

Alteration of $[Na^+]_o$. Decreasing the concentration of Na^+ in SW by over 300-fold by replacement with choline$^+$ keeping the other constituents constant (Table 1) profoundly altered the activation currents (Fig. 2). In eggs clamped at −20 mV and suspended in 1.5 mM Na^+ SW, the amplitude of I_{on} is reduced to 0, and the current is reversed during both Phases 1 and 2.

However, this decrease of Na^+ does not appreciably affect the percentage of sperm entry in eggs voltage clamped at −70 mV (Table 3). Changes of $[Na^+]_o$ affect the net current and the reversal potential for Phase 1, leading to reversal of the current from inward to outward. Hence, neither $[Na^+]_o$ nor the net current is responsible for mediating the voltage dependence.

Alteration of $[Mg^{2+}]_o$. Magnesium was reduced to 10.8 mM and raised to 260 mM by isosmotic replacement with Na^+. Decreasing $[Mg^{2+}]_o$ to 10.8 mM did not change the amplitudes of I_{on} or I_{sm} significantly, whereas increasing $[Mg^{2+}]_o$ roughly fivefold to 260 mM profoundly decreased the currents at −30 mV from −0.19 ± 0.024 nA (n = 13) and −0.37 ± 0.016 nA (n = 14) for I_{on} and I_{sm}, respectively, to −0.039 ± 0.005 nA (n = 11) and −0.079 ± 0.017 nA (n = 8). (This is approximately the reduction expected due to the concomitant decrease of $[Na^+]_o$ from 493 to 194 mM.)

Despite effects on the net inward current, decreasing or increasing $[Mg^{2+}]_o$ approximately fivefold did not have a significant effect on sperm entry for eggs clamped at −30 mV (Table 4). Therefore neither the magnitude of the current nor $[Mg^{2+}]_o$ mediates the voltage dependence of sperm entry.

Alteration of $[Ca^{2+}]_o$. In contrast to $[Na^+]_o$ and $[Mg^{2+}]_o$, a decrease or increase of $[Ca^{2+}]_o$ of comparable magnitude profoundly altered the percentage of sperm that entered. In this series of experiments, $[Ca^{2+}]_o$ was decreased below the regular $[Ca^{2+}]_o$ in SW of 10.7 mM by isosmotic substitution of $CaCl_2$ with $MgCl_2$. Increases of $[Ca^{2+}]_o$ were accomplished by isosmotic substitution of $CaCl_2$ for NaCl (see Materials and Methods and Table 1) while $[Mg^{2+}]_o$ was maintained constant. Calcium substitution affected the maximum amplitude of the current achieved during Phase 1 (I_{sm}) but did not markedly affect the amplitude of I_{on}. As $[Ca^{2+}]_o$ increased from 1 to 89 mM, the amplitude of I_{sm} (at −20 mV) decreased from −0.35 ± 0.03 nA (n = 14) to −0.08 ± 0.002 nA (n = 46). This decrease of current was somewhat greater than that expected due to the decrease of $[Na^+]_o$ from 493 to 376 mM.

Sperm entry was increasingly inhibited when $[Ca^{2+}]_o$ was increased from 1 to 89 mM at each clamped membrane potential used (Fig. 3A). The diminished entry of sperm at increased $[Ca^{2+}]_o$ was accompanied by a decreased net inward current during Phase 1 although similar diminutions of the net current associated with substitution of $[Na^+]_o$ and/or $[Mg^{2+}]_o$ did not affect sperm entry. In the remainder of the Results, we consider the mechanism by which $[Ca^{2+}]_o$ affects sperm entry.

The smooth sigmoid curve in Fig. 3A is a theoretical curve indicating the steepness of the relationship between $[Ca^{2+}]_o$ and an equilibrium process requiring the binding of a single calcium. Note that the dependence of sperm entry on $[Ca^{2+}]_o$ at −20, −10, and 0 mV is much steeper than the theoretical curve, suggesting a higher order reaction involving more than one calcium. At −30, −50, and −70 mV, the dependence on $[Ca^{2+}]_o$ was not as steep.

The relationship between V_m and $[Ca^{2+}]_o$'s inhibition of sperm entry is accessible using plots of the voltage dependence of sperm entry at different $[Ca^{2+}]$s (Fig. 3B). Sperm entry similarly decreases as V_m is held more negative for each $[Ca^{2+}]_o$ examined. The V_m at which sperm entry decreases to 50% ($V_{1/2}$), occurs at decreasingly negative values as $[Ca^{2+}]_o$ is increased. At the highest $[Ca^{2+}]_o$ examined (89 mM), sperm entry is almost completely suppressed at all potentials examined. To state these findings of Figs. 3A and 3B in another way, for sperm entry, increasing $[Ca^{2+}]_o$ with V_m held constant duplicates the effect of hyperpolarizing the egg's V_m with $[Ca^{2+}]_o$ held constant.

At all $[Ca^{2+}]_o$ and all V_ms examined (more negative than +18 mV), a successful sperm attachment is invariably associated with generation of an EPR. The same types of EPRs elicited by entering and nonentering sperm, and the same progression of change in type of EPR as the V_m becomes more negative, are observed at each of the $[Ca^{2+}]_o$ examined (see Background). As $[Ca^{2+}]_o$ is decreased below 10.7 mM, the progression of change in the types of EPRs elicited by entering and nonentering sperm continues al-

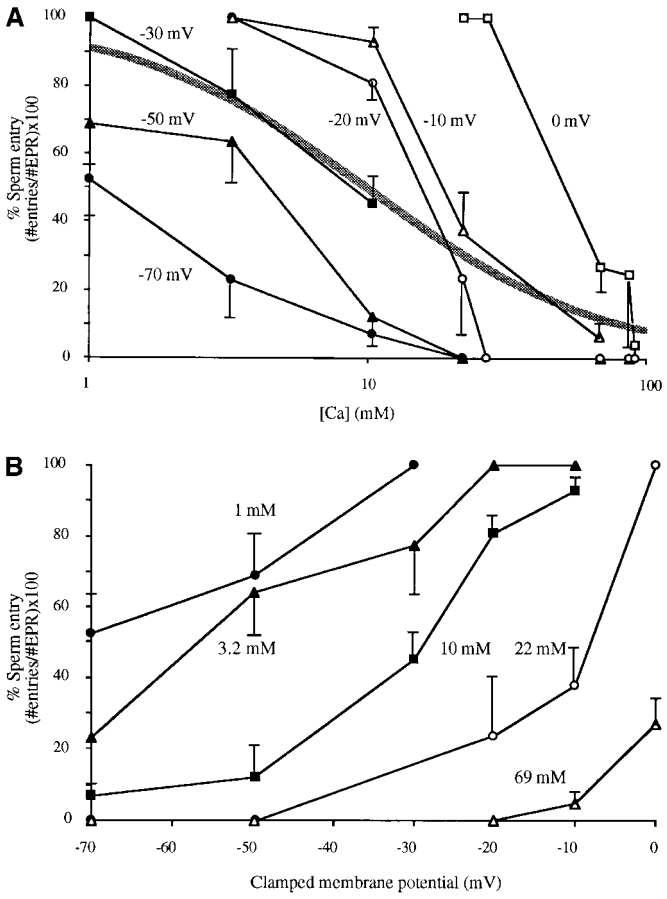

FIG. 3. In voltage-clamped eggs, the percentage of sperm entry is diminished when $[Ca^{2+}]_o$ is increased in artificial SW mixtures, but the percentage of sperm entry is promoted when $[Ca^{2+}]_o$ is decreased. (A) Percentage sperm entry as a function of $[Ca^{2+}]_o$. Eggs were clamped at 0 mV (open squares), −10 mV (open triangles), −20 mV (open circles), −30 mV (filled squares), −50 mV (filled triangles), or −70 mV (filled circles). The broad shaded line is a Michaelis–Menten function with a K_m of 10 mM (single Ca^{2+} site). (B) Percentage sperm entry as a function of the egg's clamped V_m. Eggs were suspended in 1 mM (filled circles), 3.2 mM (filled triangles), 10.7 mM (filled squares), 22.1 mM (open circles), or 69.3 mM (open triangles) $[Ca^{2+}]_o$ SW. Error bars, ±SE.

though it is shifted to more negative values (to the left along the voltage axis, see Fig. 3B) and when $[Ca^{2+}]_o$ is increased, the change in the types of EPRs is shifted to more positive V_ms.

High $[Ca^{2+}]_o$ Precludes Sperm Entry into Eggs That Are Not Voltage Clamped

The profound suppression of sperm entry into eggs clamped at 0 mV when suspended in the media containing 69 and 89 mM Ca^{2+} (see Figs. 3A and 3B) suggested that for unclamped eggs suspended in the same media (resting V_m approx −80 mV) sperm entry would also be severely suppressed even if the membrane potential was not voltage clamped. Indeed, this proved to be the case. We recorded V_m but did not maintain it constant by voltage clamp (Chambers and McCulloh, 1990) from eggs bathed in 69 mM Ca^{2+}. Upon insemination of the eggs, the predominant EPRs were those elicited by sperm which did not enter the eggs, namely, "sperm transient potentials" or "modified activation potentials." These responses comprised an abrupt depolarization that was sustained for several seconds followed by an abrupt repolarization to the resting V_m of the egg. These responses are the voltage homologue, respectively, of sperm transient currents or modified activation currents observed in voltage clamped eggs, but with superimposed action potentials. (Of course, in voltage clamped eggs, action potentials do not occur.) Failure of sperm entry for eggs bathed in high Ca^{2+} signified that the near-normal depolarizations caused by sperm interactions with these eggs were not sufficient to permit sperm entry.

We considered two possible causes for these shifts of voltage dependence for sperm entry and type of EPR by changes of $[Ca^{2+}]_o$:

(1) Ca^{2+} change of the local field of the voltage sensor either: (a) by binding at a specific Ca^{2+} site very nearby or (b) by nonspecific adsorption to the fixed negative charges on the surface of the egg's membrane or

(2) a specific transmembrane Ca^{2+} flux (whereby Ca^{2+} senses the field of the membrane as it passes through the membrane).

The shift for $V_{1/2}$ caused by changing $[Ca^{2+}]_o$ is more than 40 mV for the change of $[Ca^{2+}]_o$ from 1 to 10 mM. This

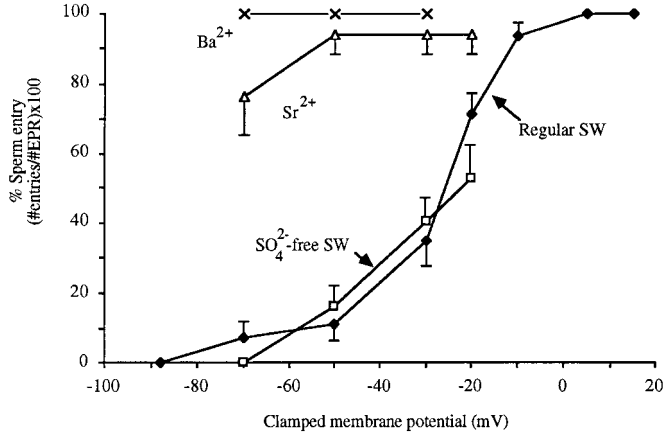

FIG. 4. In voltage-clamped eggs the percentage of sperm entry is markedly promoted by substituting Ba^{2+} (x) or Sr^{2+} (open triangles) for Ca^{2+} in SO_4^{2-}-free SW. Controls, 10.7 mM Ca^{2+} in SO_4^{2-}-free SW (open squares) or regular SW (filled diamonds). Error bars, ±SE. Sperm entry was confirmed by observing sperm asters.

magnitude of shift exceeds the maximum possible (29 mV/10-fold change of concentration) for an effect of Ca^{2+} on the local field (Frankenheuser and Hodgkin, 1957). This suggests that alteration of the local field would not be sufficient to account for the magnitude of $[Ca^{2+}]_o$'s effects.

Substitution of Ca^{2+} by Sr^{2+} or Ba^{2+} Relieves the Inhibition of Sperm Entry

Fixed negative charges on the external surface of the egg's membrane would be expected to adsorb Ca^{2+}, Ba^{2+}, and/or Sr^{2+} relatively equally and nonspecifically. Therefore we substituted Ba^{2+} or Sr^{2+} for Ca^{2+} in SO_4^{2-}-free SW (Fig. 4) as another test of the nonspecific adsorption effect. The control for these experiments was SO_4^{2-}-free SW containing 10.7 mM Ca^{2+}.

Sr^{2+} or Ba^{2+} were substituted for Ca^{2+} on an equimolar basis. This reduced the amplitude of the currents during Phase 1 of all three types of EPR. At −30 mV the amplitude of I_{on} was decreased to -0.025 ± 0.008 nA ($n = 15$) in Sr^{2+} SW and to -0.027 ± 0.007 nA ($n = 2$) in Ba^{2+} SW. For comparison, in sulfate-free SW the amplitude of I_{on} was -0.21 ± 0.011 nA ($n = 36$), not significantly different from that in normal SW, -0.19 ± 0.024 nA ($n = 13$). Likewise, at −30 mV, I_{sm} was decreased to -0.15 ± 0.056 nA ($n = 15$) in Sr^{2+} SW and to −0.36 nA ($n = 1$) in Ba^{2+} SW. In sulfate-free SW, the amplitude of I_{sm} was -0.63 ± 0.029 nA ($n = 25$), slightly larger than the value in normal SW (-0.37 ± 0.016 nA, $n = 14$). The reduction in current cannot be attributed to changes in $[Na^+]_o$; they may be caused by the decrease of $[Ca^{2+}]_o$ and/or by the Sr^{2+} or Ba^{2+}.

Sperm entry in the SO_4^{2-}-free control solution was not significantly different from that in regular SW (Fig. 4). The curves show that reducing $[Ca^{2+}]_o$ to 0 mM (no chelators were used so this is only nominally 0 mM) by replacement with Ba^{2+} or Sr^{2+} markedly enhances sperm entry at V_ms at which sperm entry is impaired in control seawater. The increase of sperm entry is even more than that observed in 1 mM Ca^{2+} (compare Ba^{2+} and Sr^{2+} curves in Fig. 4 with curve for 1 mM Ca^{2+} in Fig. 3B). Therefore, neither Ba^{2+} nor Sr^{2+} can substitute completely for Ca^{2+} with regard to its inhibition of sperm entry at negative V_ms. The failure of Ba^{2+} or Sr^{2+} to sustain the block of sperm entry at negative membrane potentials suggests that Ca^{2+}'s mode of action is more specific than as a nonspecific adsorbed divalent cation affecting surface potential.

Ca^{2+} Channel Blocking Agents Relieve the Inhibition of Sperm Entry

Since Ca^{2+}'s shift of the voltage dependence is so large and since Ca^{2+}'s action is not nonspecifically mimicked by other divalent ions, we considered the two remaining specific modes of action for Ca^{2+}: specific binding that directly alters the electrical field near a voltage sensor or specific Ca^{2+} flux. In order to distinguish between binding and flux, we examined the possibility that the voltage dependence of sperm entry is mediated by Ca^{2+} influx by suspending eggs in SW containing Ca^{2+} channel blocking agents. V_m was clamped at −30 or at −50 mV and the egg was inseminated by adding sperm directly to the suspension medium. Agents from three different classes of organic Ca^{2+} channel antagonists, the phenylalkylamine verapamil, the benzothiazipine diltiazem, and the dihydropyridines nifedipine and nimodipine, and the inorganic blockers Cd^{2+} and Ni^{2+} were examined.

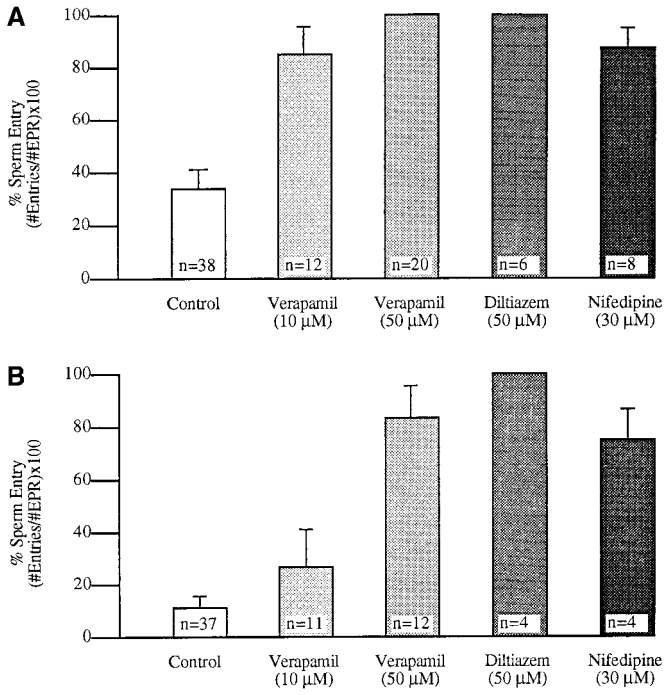

FIG. 5. The organic Ca^{2+} channel blocking agents promote sperm entry. (A) Eggs clamped at −30 mV. (B) Eggs clamped at −50 mV. Error bars, ±SE. Sperm entry in monospermic eggs was confirmed by observing sperm asters.

Calcium channel antagonists varied in their effects on the net current during Phase 1 of all three types of EPR. At −30 mV, 10 and 50 μM verapamil, 30 μM nifedipine, 50 μM nimodipine, and 50 μM diltiazem each diminished the amplitude of I_{on} and I_{sm} for all three types of EPR. The ionic blockers 100 μM Cd^{2+} and 100 μM Ni^{2+} reduced the amplitude of currents only minimally during Phase 1.

The effects of these agents in promoting sperm entry (Figs. 5A and 5B) are similar to decreasing $[Ca^{2+}]_o$. For example, verapamil at 50 μM and nifedipine at 30 μM promote sperm entry to approximately the same extent as decreasing $[Ca^{2+}]_o$ in regular SW 10-fold (see Fig. 3B, compare curve for 10.7 mM $[Ca^{2+}]_o$ with curve for 1.0 mM $[Ca^{2+}]_o$ for eggs clamped at −30 mV and at −50 mV, respectively). The effectiveness of diltiazem at 50 μM

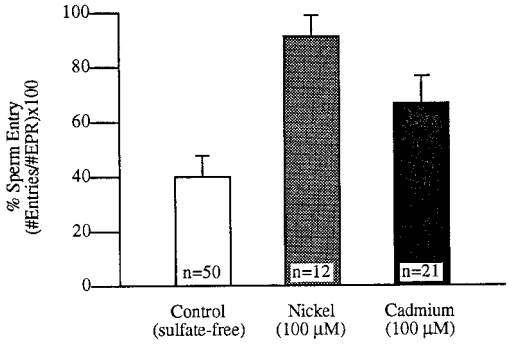

FIG. 6. The heavy metal Ca^{2+} channel blocking agents Cd^{2+} and Ni^{2+} promote sperm entry. Eggs were clamped at −30 mV. Error bars, ±SE. Sperm entry was confirmed by observing sperm asters.

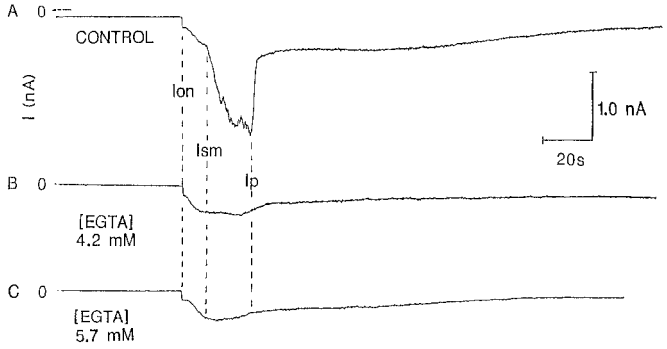

FIG. 7. Microinjection of the Ca^{2+} chelator EGTA obliterates the major peak current, I_p. Eggs suspended in SW were voltage clamped at −20 mV. (A) Control egg microinjected (~1% egg volume) with vehicle consisting of 0.35 M K_2SO_4 and 10 mM Pipes, pH 6.9, in pipette. (B and C) Eggs microinjected (~1% egg volume) with 500 mM KEGTA.

approaches that of decreasing $[Ca^{2+}]_o$ in SW to nominal 0 (see Fig. 4, substitution of Ba^{2+} or Sr^{2+} for $[Ca^{2+}]_o$). Nimodipine (50 μM) resulted in sperm entry in 4 of 4 EPRs in eggs in which V_m was maintained at −30 mV.

The heavy metal Ca^{2+} channel blocking agents (100 μM) made up in SO_4^{2-}-free SW also promoted sperm entry in eggs clamped at −30 mV (Fig. 6). Ni^{2+}, which is more effective than Cd^{2+}, enhances sperm entry to about the same extent as decreasing $[Ca^{2+}]_o$ in regular SW 10-fold (see Fig. 3B, compare curve for 10.7 mM Ca^{2+} with curve for 1.0 mM Ca^{2+} for eggs clamped at −30 mV). These experiments were necessarily limited in scope, since the heavy metals adversely affect sperm motility. For this reason Co^{2+} could not be used.

All blockers uniformly yielded similar effects on sperm entry, increasing the incidence of sperm entry at −30 and −50 mV. The disparity between the blockers' effects on net currents highlights the previous conclusion that net current does not regulate sperm entry. However, the net current doesn't necessarily reflect Ca^{2+} influx. In fact, this is the simplest explanation of the data. The wide array of Ca^{2+} channel antagonists used and their universal effect (relief of the voltage-dependent failure of sperm entry) suggest that Ca^{2+} flux through Ca^{2+} channels is intimately associated with the block of sperm entry at negative membrane potentials. We currently do not know the identity of the Ca^{2+} channels which, when blocked, affect sperm entry nor whether these channels are located in the plasma membrane of the egg or of the fertilizing sperm.

Microinjection of Ca^{2+} Chelators Relieves the Inhibition of Sperm Entry

If calcium influx mediates the block of sperm entry, then Ca^{2+} may act at an intracellular site. This possibility was examined by microinjecting a calcium chelator, EGTA or BAPTA, into eggs suspended in regular SW (volumes injected ~1% of egg). The effects were compared to controls microinjected with the identical solution, but without the Ca^{2+} chelator. Microinjection of EGTA or BAPTA had no significant effect on the amplitude of currents during Phase 1 of all three types of EPR; however, it did affect Phase 2. EGTA or BAPTA was microinjected at intracellular concentrations which fully obliterated Phase 2 of the activation current, namely, 0.30 to 7.6 mM for EGTA and 0.30 to 0.87 mM for BAPTA. Phase 2, which includes I_p, the inward current peak of the second phase of the activation current, is a nonspecific cation current (Fig. 7A), activated by an

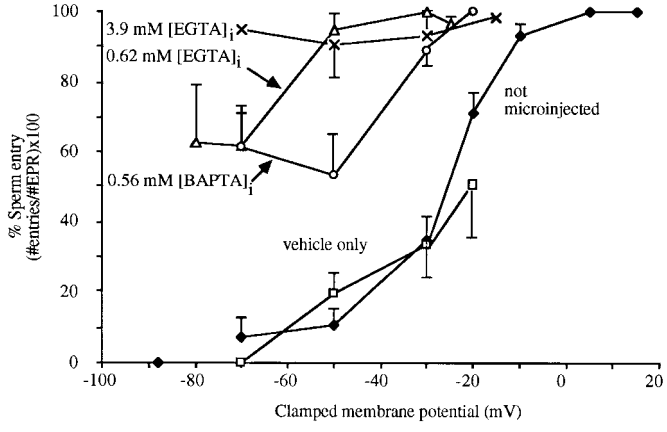

FIG. 8. In voltage-clamped eggs the microinjection of EGTA or BAPTA markedly promotes the percentage of sperm entry. Eggs were suspended in regular SW (10.7 mM Ca^{2+}) with intracellular concentration of EGTA 0.62 mM (open triangles) or 3.9 mM (x) or BAPTA 0.56 mM (open circles); control eggs were microinjected with vehicle only (open squares) or not microinjected (filled diamonds).

increase of $[Ca^{2+}]_i$ (Halliwell and Whitaker, 1988; Chambers and McCulloh, 1990; Swann *et al.*, 1992; Mohri *et al.*, 1995). The obliteration of Phase 2 by microinjection of the Ca^{2+} chelators indicated that the increase in the level of $[Ca^{2+}]_i$ normally triggered by the sperm had been reduced below the threshold required to open the cation channels in the inseminated egg's membrane (Swann *et al.*, 1992). This effect is shown in Figs. 7B and 7C, in comparison to a control egg (Fig. 7A) microinjected with vehicle only. When Phase 2 is eliminated, invariably the fertilization envelope does not elevate, the hyaline layer is not secreted, the sperm monaster does not develop, and cleavage fails to occur. As long as active sperm are present, sperm continue to attach to and enter the egg, superimposing new EPRs on the electrical record.

The effects of microinjecting EGTA on the voltage dependence of sperm entry were compared for two different concentration ranges. The first range comprises the lowest concentrations of EGTA required to abrogate Phase 2, namely 0.30 to 0.92 mM, mean 0.62 ± 0.06 mM (n = 10). The second range includes those concentrations of EGTA which were in excess of that required to eliminate I_p, or 2.2 to 7.6 mM, mean 3.9 ± 0.38 mM (n = 16). The results (Fig. 8) show that the voltage dependence of sperm entry of the control eggs microinjected with the vehicle only is not significantly different from that of uninjected control eggs. Sperm entry was markedly facilitated at negative V_ms for eggs microinjected with EGTA. The relief of inhibition was dependent on $[EGTA]_i$, the effect being more pronounced for microinjections of EGTA at the higher final intracellular concentration range (mean 3.9 mM) than at the lower final concentrations (mean of 0.62 mM). The magnitude of the effect of microinjecting EGTA at the higher concentration range in promoting sperm entry is similar to that observed when eggs are suspended in nominal Ca^{2+}-free SW by substituting Sr^{2+} or Ba^{2+} for Ca^{2+} (compare Fig. 8 with Fig. 4).

An interesting aspect of the EGTA microinjection experiments is that the amplitude of the sperm induced I_{on} and of the early inward current during the first 12 sec is the same, at corresponding V_ms, as for the controls injected with the K_2SO_4 vehicle only. This was true whether the eggs had been microinjected with EGTA in the lower, or in the higher concentration range. The absence of an effect on the amplitude of inward current suggests that microinjections of EGTA have no effect on Ca influx during this early period and hence EGTA's sole effect on sperm entry is the consequence of lowering $[Ca^{2+}]_i$. (Recall that it is during the first 12 sec after I_{on} when the changes occur that are responsible for determining whether or not sperm entry will occur).

The effect of microinjecting BAPTA at a final average intracellular concentration of 0.56 ± 0.06 mM (n = 14) on sperm entry was also examined. This concentration range is the minimum required to fully eliminate activation of the egg. The results were not significantly different from those obtained by microinjecting EGTA at comparable concentration (see Fig. 8). In several experiments BAPTA was microinjected at concentrations insufficient to fully suppress I_p and activation of the egg. Even at these lower concentrations sperm entry into eggs clamped at −30 mV is substantially facilitated, compared to controls.

DISCUSSION

There is a calcium- and voltage-dependent block to sperm entry into sea urchin eggs at negative membrane potentials. Sperm incorporation diminished at all negative holding potentials when $[Ca^{2+}]_o$ was increased (Fig. 3A) and at all $[Ca^{2+}]_o$ when V_m was held increasingly negative (Fig. 3B). The voltage dependence for inhibition of sperm entry was shifted to more hyperpolarized potentials if $[Ca^{2+}]_o$ was reduced and the half-maximal $[Ca^{2+}]_o$ was shifted to lower concentrations if the cells were hyperpolarized.

The voltage-dependent inhibition of sperm entry was relieved by reduced $[Ca^{2+}]_o$. The relief was mimicked by:

(1) substitution of Ba^{2+} or Sr^{2+} for Ca^{2+},
(2) use of any of four classes of Ca^{2+} channel blockers, and
(3) cytoplasmic injection of Ca^{2+} chelators.

The simplest explanation of all the data is that calcium influx mediates the voltage-dependent block of sperm entry, responding to the membrane's electrical field as it traverses the plasma membrane to accumulate in the cytoplasm. Calcium then acts at an unidentified site within the egg to inhibit sperm incorporation.

Effects of Changing Voltage and Calcium

$[Ca^{2+}]_o$ ranging from 1 to 90 mM inhibited sperm entry but wide-ranging substitutions of Na^+ and Mg^{2+} had no effect on this inhibition. The voltage dependence of sperm entry suggests that there is a sensor, a charged or dipolar entity, within the plasma membrane. Equivalent diminutions of sperm entry were produced by either increasing $[Ca^{2+}]_o$ or maintaining the V_m more negative, showing the interchangeability of $[Ca^{2+}]_o$ and V_m. Raising $[Ca^{2+}]_o$ or hyperpolarizing the cell increases the driving force on calcium ions and would be expected to increase their flux through open channels.

The shift of the voltage dependence relationship with $[Ca^{2+}]_o$ was greater than 40 mV/10-fold change, greater than the maximum theoretically possible (29 mV/10-fold change) for an effect on surface charge. Experimentally surface charge effects typically produce only an 8–15 mV/10-fold change (Frankenheuser and Hodgkin, 1957; Hille, 1992). Therefore, the mechanism of the Ca^{2+} shift of the voltage dependence of inhibition of sperm entry is not by nonspecific charge shielding.

Ion substitution experiments also suggest that nonspecific surface adsorption is not the mechanism by which Ca^{2+} acts. Complete substitution of Ba^{2+} or Sr^{2+} for Ca^{2+}

allowed sperm entry instead of mimicking Ca^{2+}'s inhibitory effect. Sperm entry wasn't affected by a 25-fold increase of $[Mg^{2+}]_o$ with $[Ca^{2+}]_o$ maintained constant. Thus Ca^{2+} specifically inhibits sperm entry either by Ca^{2+} influx or by specific Ca^{2+} binding at sites near the voltage sensor.

Ca^{2+}'s Specific Action Involves Influx and Cytoplasmic Accumulation

The experiments with Ca^{2+} channel antagonists and/or intracellular Ca^{2+} chelators show that Ca^{2+}'s mechanism involves influx and accumulation in the cytoplasm, respectively. The size of either effect was equivalent to a 10-fold or greater reduction of $[Ca^{2+}]_o$, nearly eliminating the inhibition of sperm entry within the range of potentials examined (−70 to −10 mV).

Injection of neomycin inhibits sperm entry in eggs clamped at −20 mV which show prolonged type II EPRs. At more hyperpolarized potentials the EPR duration shortens but remains longer than the EPRs of uninjected eggs (Swann *et al.,* 1992). Neomycin also increases the concentration of calcium required to produce half-maximal exocytosis in isolated fragments of plasma membranes from sea urchin eggs (McLaughlin and Whitaker, 1987). These two effects of neomycin may have different origins because neomycin acts similarly to calcium in preventing sperm entry but antagonizes the action of calcium on exocytosis.

Calcium Channel Antagonists

Four classes of Ca^{2+} channel antagonists (phenylalkylamines, dihydropyridines, benzothiazipines, and ionic antagonists Ni^{2+} and Cd^{2+}) each relieved the inhibition of sperm entry associated with negative V_m. This suggests that Ca^{2+} channels participate in the Ca^{2+}-dependent inhibition of sperm entry. The simplest interpretation is that the antagonists relieve the inhibition by blocking Ca^{2+} influx.

The possibility that these are nonselective effects of these materials is unlikely because they enhanced sperm entry. We saw fewer EPRs in seawater containing Ca^{2+} antagonists; fertilization was less efficient. However, once an EPR occurred, sperm entry was more frequent in the presence of the antagonists. Their chemical dissimilarity also makes it unlikely that they would produce similar nonspecific effects.

The effects of all of these antagonists were similar to decreasing $[Ca^{2+}]_o$. The experiments do not distinguish among types of channels. Either all the antagonists are effective in blocking a single channel type or there are several different types of Ca^{2+} channels and/or nonspecific cation channels which contribute to regulation of sperm entry.

We also do not know whether the channels are in the egg's membrane near the site of sperm–egg fusion or in the sperm's membrane. One possibility is that calcium channels identified in sea urchin sperm plasma membranes (Guerrero and Darszon, 1989; Lievano *et al.,* 1990) are the channels involved. The influx of Ca^{2+} is thought to be a minor component of the net inward current during Phase 1 of all EPRs. Probably the Ca^{2+} influx is near the sperm because all the current that flows into the egg during Phase 1 is localized there (McCulloh and Chambers, 1992). It seems reasonable to suggest that Ca^{2+}'s site of influx is near its site of action: the region of the egg near the sperm that is inhibited from entering.

We applied the organic Ca^{2+} channel blockers in high concentrations compared to those effective in mammalian systems (20–100 nM; Hagiwara and Byerly, 1981). Higher concentrations may be needed to block Ca^{2+} channels in invertebrate systems (Hille, 1992). Bean (1984) has shown that the effective inhibitory concentrations of dihydropyridines is higher in partially depolarized cells compared to fully depolarized. Our eggs were not fully depolarized but were maintained at −30 or −50 mV. Kazazoglou *et al.* (1985), using the same three organic Ca^{2+} antagonists, found that a range of concentrations (10 to 50 μM) similar to what was used in our experiments was needed to inhibit by 50% the acrosome reaction of sea urchin sperm (also a Ca^{2+}-dependent process), presumably by blocking Ca^{2+} influx.

Calcium Chelators in Cytoplasm

Microinjection of the Ca^{2+}-chelating agents EGTA or BAPTA relieved the inhibition of sperm entry into eggs suspended in regular SW and clamped at negative V_ms. Intracellular concentrations of chelator that suppressed the Ca^{2+}-dependent Phase 2 of the activation current without significantly changing Phase 1 were effective (Fig. 7). This indicates a cytosolic Ca^{2+} site. Intracellular application of a chelator wouldn't be expected to alter the extracellular surface charge.

When Ba^{2+} or Sr^{2+} was substituted for $[Ca^{2+}]_o$, the block of sperm entry was relieved, similar to the microinjection of Ca^{2+} chelators. This does not distinguish whether the substituted divalent cations fail to pass through Ca^{2+} channels or fail to activate an intracellular site. Influx and cytosolic effects exhibit different divalent selectivities during smooth muscle contraction (Fay *et al.,* 1992) and during synaptic exocytosis (Augustine and Eckert, 1984). In both systems, the cytosolic events occur poorly when $[Ca^{2+}]_o$ is decreased by substitution. In sea urchin sperm, calcium influx is blocked by millimolar concentrations of ionic blockers Cd^{2+} (Lievano *et al.,* 1990) and Ni^{2+} (Reynaud *et al.,* 1993) and by the dihydropyridine nisoldipine (Guerrero and Darszon, 1989). Ba^{2+} and Sr^{2+} were more permeant than Ca^{2+} in at least one Ca^{2+} channel (Lievano *et al.,* 1990). Sr^{2+} was capable of passing through the sperm plasma membrane and acting in the cytosol, inducing the sperm's acrosome reaction (Guerrero *et al.,* 1998).

The dependence of the prevention of sperm entry on $[Ca^{2+}]_o$ is not simple. The curves at −20, −10, and 0 mV (Fig. 3A) are steeper than expected for a single Ca^{2+} site.

Thus the inhibition of sperm entry may involve cooperative binding of more than one calcium ion. This is speculative because the relationship between $[Ca^{2+}]_o$ and the amount of Ca^{2+} reacting intracellularly is not known. The local concentration near the site of calcium entry may be much higher than that suggested by the small currents and the large volume of the egg. In squid synapses Sugimori *et al.* (1994) have reported calcium concentration microdomains of about 300 μM.

CONCLUSION

The voltage-dependent exclusion of sperm by sea urchin eggs at negative membrane potentials is mediated, at least in part, by influx of calcium and action of calcium at an internal site. The influx of calcium is dependent upon both $[Ca^{2+}]_o$ and membrane potential. The strong dependence of sperm entry on $[Ca^{2+}]_o$ leads us to suggest that calcium acts at multiple cooperative Ca^{2+} binding sites. The steep dependence of sperm entry on the egg's membrane potential previously led one of us (McCulloh, 1989) to conclude that the equivalent of a minimum of three or four electronic charges traverses the entire electrical field of the plasma membrane to mediate the voltage dependence. If Ca^{2+} is the entity that mediates the voltage dependence, then more than one Ca^{2+} molecule must participate to account for the steepness. The shift of voltage dependence caused by changing the $[Ca^{2+}]_o$ shows that $[Ca^{2+}]_o$ and the mechanism mediating the voltage dependence are intimately associated. The size of the shift (~40 mV/10-fold change of $[Ca^{2+}]_o$) is consistent with a requirement for multiple Ca^{2+} molecules binding in the cytosol and thereby coupling the energy of more than one Ca^{2+} membrane crossing per unitary event of sperm exclusion. All three observations are consistent with Ca^{2+} influx and high order calcium-dependent reaction in the cytosol. Therefore, we favor this model as the simplest explanation consistent with all the observations.

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