

ELECTRICALLY MEDIATED FAST POLYSPERMY BLOCK IN EGGS OF THE MARINE WORM, *URECHIS CAUPO*

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ABSTRACT

Previous work has established that the polyspermy block in *Urechis* acts at the level of sperm-egg membrane fusion. (*J. Exp. Zool.* **196**:105). Present results indicate that during the first 5–10 min after insemination the block is mediated by a positive shift in membrane potential (the fertilization potential) elicited by the penetrating sperm, since holding the membrane potential of the unfertilized egg positive by passing current reduces the probability of sperm entry, while progressively reducing the amplitude of the fertilization potential by decreasing external Na^+ progressively enhances multiple sperm penetrations. Also, a normal fertilization potential is correlated with a polyspermy block even under conditions (pH 7) in which eggs do not develop. We have investigated the mechanism of the electrical polyspermy block by quantifying the relationship between sperm incorporation, membrane potential and ion fluxes. Results indicate that the polyspermy block is mediated by the electrical change per se and not by the associated fluxes of Na^+ , Ca^{++} , and H^+ .

KEY WORDS polyspermy prevention · membrane fusion regulation · membrane potential · ion fluxes · fertilization

Normal embryonic development requires that a single sperm nucleus unite with the egg nucleus (see reference 1 for a review of strategies for monospermic fertilization). In the eggs of *Urechis*, a significant reduction in susceptibility to polyspermic fertilization occurs within seconds after insemination (reference 38, and our results below). Paul's results (38) and ours below clearly show that this polyspermy block, rather than being absolutely effective as the term implies, operates by reducing the probability of a second sperm entry

sufficiently so that, under presumed physiological sperm-egg ratios, most eggs are monospermic. Although fertilized *Urechis* eggs become significantly more resistant to refertilization within seconds after insemination, they remain indistinguishable from unfertilized eggs by light and electron microscopy through 4 min after insemination (13), except for a rounding-out of the cell shape (the unfertilized egg has a single, large indentation). At 4 min the surface coat begins to elevate, but there is no massive exocytosis of cortical vesicles, most of which remain intact through first cleavage (13, 39). Though elevated, the surface coat is no barrier to supernumerary sperm, whose acrosomal tubules penetrate to within 100 Å of the egg plasma mem-

brane (40). Furthermore, sperm can fertilize eggs whose surface coats have been experimentally elevated before insemination (using trypsin at pH 7).¹ Thus, it is apparent that the *Urechis* polyspermy block acts by altering the egg plasma membrane to prevent fusion with supernumerary sperm. In this paper, we show that the fertilization potential, a positive shift in egg membrane potential elicited by the fertilizing spermatozoon, mediates the polyspermy block during the first 5–10 min after insemination. Such an electrical polyspermy block was first demonstrated in eggs of a sea urchin (20).

A detailed description of the fertilization potential and its ionic basis has been published separately (21); a summary essential for understanding the present research follows. A typical fertilization potential is illustrated in Fig. 5a below. Within 2 s after sperm addition, at the fastest, the membrane potential shifts from about -35 mV to a maximum near $+50$ mV with a rise time of ~ 1 s. (Presumably the interval between sperm addition and potential shift is related to the time required for the sperm to swim to and contact the egg.) After reaching the maximum, the potential falls off to a plateau at about $+30$ mV. Finally, the potential becomes negative again, returning to the level of the resting potential of the unfertilized egg ~ 9 min after the start of the rise, and continues to fall to about -60 mV by 20 min after insemination. Fertilization potentials of four monospermic eggs were not significantly different in either maximum or average amplitude from those of six dispermic eggs, although in three of the latter the rise phase had an initial step to ~ 0 mV followed by a second step to about $+50$ mV. None of the four monospermic eggs showed step rises. These results suggest that step rises are correlated with dispermy.

The fertilization potential results from an increase in Na^+ permeability and is amplified by a Ca^{++} action potential. Significant increases in $^{24}\text{Na}^+$ and $^{45}\text{Ca}^{++}$ influxes accompany the fertilization potential (see also reference 23). Since these same events can be elicited by trypsin without insemination (see also reference 23), the ion channels responsible must pre-exist within the egg membrane. During monospermic fertilization, only a fraction of the available Na^+ channels is opened; this conclusion is based on the observation

that Na^+ influx is directly proportional to the number of penetrating sperm when polyspermy is induced either by high sperm-egg ratios or by insemination in low Na^+ seawater, which reduces the amplitude of the fertilization potential (see also Fig. 3 in this paper). Therefore we conclude that the Na^+ channels are sperm-gated (i.e., each sperm opens a set of Na^+ channels). Ca^{++} influx, on the other hand, occurs primarily via a membrane potential-gated channel, the Ca^{++} action potential, and is independent of additional sperm penetrations in normal seawater. However, in 50 mM Na^+ seawater where the amplitude of the fertilization potential remains below the voltage threshold ($+10$ mV) for the action potential, some residual Ca^{++} influx occurs and this is proportional to the number of penetrating sperm.

In addition to demonstrating that the early phase of the polyspermy block in *Urechis* eggs is electrically mediated, we have further explored the mechanism of the block. It is possible to vary ion fluxes independently of membrane potential by altering external ion concentrations, or by varying the concentration of sperm used for insemination in low Na^+ seawater (see above); thus, we have been able to quantify the relationship between sperm incorporation, membrane potential and ion fluxes. Our results indicate that the potential change per se prevents membrane fusion during the early phase of the polyspermy block, since the eggs' susceptibility to polyspermy during this period (the first 5–10 min after insemination) varies directly with the amplitude of the membrane potential but not with the fluxes of specific ions.

MATERIALS AND METHODS

Procedures for maintaining *Urechis* adults and for obtaining and handling gametes have been described (12). Eggs were incubated at 17°C in either natural or artificial seawater (486 mM NaCl, 10 mM KCl, 27 mM MgCl_2 , 29 mM MgSO_4 , 10 mM CaCl_2 , 2.4 mM NaHCO_3 ; pH 8). Unfertilized *Urechis* eggs are in the diplotene-diakinesis stage of meiotic prophase; morphological events occurring after insemination are: rounding out (0–4 min at 17°C), surface coat elevation (begins at 4 min), germinal vesicle breakdown (begins at 6 min), first and second polar body formation (35 and 45 min), and cleavage (90 min). Rounding out, surface coat elevation, and germinal vesicle breakdown are referred to collectively as "activation."

Microelectrode Measurements

The methods used are described in detail by Jaffe et al. (21). To maintain constant temperature (17°C), ex-

¹ Paul, M., University of Victoria, British Columbia. Personal communication.

periments were done either in a controlled temperature room or with a water-cooled microscope stage. Eggs were immobilized in small scratches on the bottom of a plastic petri dish. Microelectrodes with resistances of 30–60 M Ω were filled with 3 M KCl. One electrode was used for both recording and passing current; the IR drop across the electrode was electronically balanced by means of a bridge circuit (Biodyne Electronics Laboratory, Santa Monica, Calif., Model AM-2; or W-P Instruments, Inc., New Haven, Conn., Model M4-A). Applied currents were no more than 3×10^{-10} A to ensure constant electrode resistance. The bath was grounded through a seawater-agar bridge to prevent contact of eggs with Ag⁺ ions, which even at 2×10^{-10} M activate *Urechis* eggs. Membrane potential and applied current were recorded on a chart recorder (Gould, Inc., Cleveland, Ohio, Model 220). Eggs were inseminated in the recording chamber by adding drops of sperm suspension. Except as noted, final sperm concentrations were $\sim 10^6$ – 10^7 per ml (~ 1 :30,000 to 1:3,000 final dilution). In some experiments, we exchanged the solution bathing the eggs by perfusion as described by Jaffe et al. (21).

Assay for Sperm Penetration

The method (modified from Paul [38]) is described in detail by Gould-Somero et al. (14). At 25–30 min after insemination in eggs which have activated (undergone germinal vesicle breakdown), the nuclei of penetrated sperm have decondensed and are easily seen in whole eggs by phase contrast microscopy, after fixation in 3:1 ethanol:acetic acid and clearing in 60% acetic acid (Fig. 1). In experiments employing very dense sperm suspensions, externally bound sperm which would otherwise obscure the cytoplasm are removed by prior fixation in 4% neutral formaldehyde followed by washing in 0.5 M glucose to disperse the surface coat with adherent sperm. Eggs are then transferred to 3:1 fixative. In highly polyspermic eggs, contrast was enhanced with lactic-acetic orcein, and counting of sperm nuclei was facilitated by flattening the fixed eggs under a coverslip and using a micrometer grid. In experiments with electrode-penetrated eggs, ten or more of the neighboring unimpaled eggs were fixed to score for sperm penetration and the other neighboring eggs were cleared away from the vicinity of the impaled egg, which was then gently removed from the electrode and fixed. When membrane potential was not to be recorded, 3 ml of a 1:100 egg suspension was inseminated with a given sperm dilution and the entire sample was fixed as described above. We usually scored at least 100 eggs per sample; occasionally in samples with over 25 sperm per egg only 40 eggs were scored.

Under some conditions (e.g., pH 7) eggs do not activate although they are penetrated by sperm; the sperm nuclei remain condensed and are difficult to distinguish from those of externally bound sperm (see reference 14). Therefore, we activated these eggs (to cause the sperm

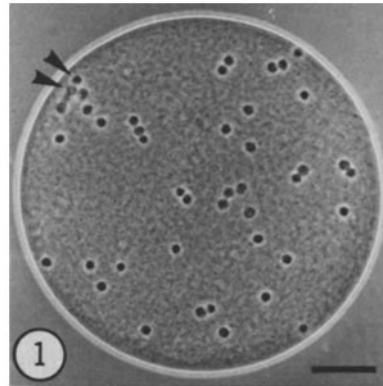


FIGURE 1 Phase contrast photomicrograph of a highly polyspermic egg inseminated in 120 mM Na⁺ seawater and fixed as described in Materials and Methods. Individual decondensed sperm nuclei are indicated by arrows (44 are visible in this optical section). Scale equals 20 μ m.

nuclei to decondense) with high K⁺ seawater, pH 8 (usually 370 mM K⁺, 120 mM Na⁺), in which eggs activate synchronously and with the normal time course through germinal vesicle breakdown and sperm nuclear decondensation.

Sperm Inactivation

Since *Urechis* sperm retain their fertilizing capacity for 30 min or more after they are diluted into seawater, it was necessary to inactivate them when eggs were to be exposed to "pulses" of sperm for limited periods. Usually, sperm were inactivated by high K⁺ seawater (370–420 mM K⁺); occasionally, they were inactivated by a 15-s exposure to 0.001% sodium dodecyl sulfate in seawater. Inactivation of sperm in high K⁺ seawater was demonstrated as follows. When a sperm suspension was diluted into high K⁺ seawater, and eggs were added within 3 s, none of the eggs was penetrated by sperm.

Tracer Uptake Studies

Methods are described in detail by Jaffe et al. (21). ²⁴Na⁺ and ⁴⁵Ca⁺⁺ (New England Nuclear, Boston, Mass.) were used at 7.5 and 2–4 μ Ci/ml, respectively. Eggs diluted 1:50 (vol/vol; 1 ml of this suspension contains $\sim 14,000$ eggs as determined by counting) were incubated in a shallow layer on the bottom of a beaker. Final dilutions of sperm are noted in figure legends. Samples were washed (10 ml each wash) by centrifuging and resuspending in chilled (4°–10°C) seawater. ²⁴Na⁺-labeled samples were washed four times (total wash time, 10–15 min) and ⁴⁵Ca⁺⁺ samples, seven times (total wash time, 15–20 min). After washing, eggs containing ²⁴Na⁺ were dispersed in 2 ml of 0.5% sodium dodecyl sulfate (in deionized water), and their radioactivity was measured in a Nuclear-Chicago gamma radiation counter

(Nuclear-Chicago Corp., Des Plaines, Ill.). Washed eggs containing $^{45}\text{Ca}^{++}$ were collected by suction onto Whatman glass fiber GFA filters which were placed into scintillation vials with 0.4 ml of Protosol:water (9:1) to disperse them. 10 ml of scintillation fluid (4 g 2,5-diphenyloxazole (PPO), 0.05 g 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) per liter toluene) was added to each vial and radioactivity was measured in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). For determining specific activity in the incubation mixtures, triplicate 10- μl aliquots of the supernatant seawater were counted under the same conditions.

Calculations and Statistics

It is frequently useful to describe fertilization potentials in terms of their average amplitude during a given time period. The average amplitude of a fertilization potential during the first min after insemination was calculated by averaging the potential at $T = 0, 15, 30, 45,$ and 60 s after the initial rise. An average plateau potential omits the first 15 s of the activation potential and is calculated by averaging the potential at $T = 0.25, 1, 2, 3, 4,$ and 5 min after the initial rise. All calculations of means in the text are followed by the standard deviation. The standard error of the mean can easily be calculated ($\text{SEM} = \text{SD} + \sqrt{n}$) to compare the means of two groups.

RESULTS

Three lines of evidence establish that the positive-going fertilization potential constitutes an electrical block to polyspermy. First, sperm incorporation is inhibited when the membrane potential of an unfertilized egg is held positive; second, polyspermy is induced when the potential is held negative; and third, susceptibility to polyspermy is correlated with fertilization potential amplitude (see also reference 20).

Positive Potential Induced by Outward Current Inhibits Sperm Penetration

When outward current ($+2$ to $+3 \times 10^{-10}$ A) is applied through an intracellular electrode to set the membrane potential of an unfertilized egg more positive than $+25$ mV (Fig. 2a), then sperm are added, the probability of sperm penetration is significantly reduced. In seven of ten "acceptable" eggs (criteria are listed in footnote to Table I) no sperm penetrated (Table I). Control experiments (Fig. 2c) showed that application of outward current ($+2$ to $+3 \times 10^{-10}$ A) just before, but not during, sperm addition does not prevent sperm penetration (7/7 eggs; see Table I). These controls

(and those in reference 21) also demonstrate that electrode penetration itself does not prevent sperm entry. When the membrane potential of unfertilized eggs is set at about -5 mV by outward current ($+0.5$ to $+1 \times 10^{-10}$ A, Fig. 2b), then sperm are added, the probability of sperm penetration is also reduced: in three of six eggs no sperm entered (Table I). However, sperm elicited a fertilization potential in all six eggs; therefore, under these experimental conditions, nonpenetrating sperm are able to trigger an electrical response in the egg.

Negative Potential Induced by Decreasing External Na^+ Causes Polyspermy

Attempts to induce polyspermy by passing inward current to reduce the amplitude of the fertilization potential were unsuccessful because the egg membrane resistance is too low during the first 2 min after insemination. Inward currents as large as -3×10^{-10} A failed to reduce the potential below 0 mV (Fig. 2d). If much larger currents are passed through the single electrode, the membrane potential cannot be measured accurately due to nonlinear effects of large currents on electrode resistance. Therefore, since Na^+ is the major current-carrying ion during the fertilization potential (21), we reduced the amplitude of the fertilization potential by decreasing external Na^+ (a ten-fold reduction in external Na^+ reduces the average amplitude of the fertilization potential by ~ 50 mV; reference 21, and Fig. 3 insert). When eggs are inseminated in decreasing concentrations of Na^+ , they become increasingly polyspermic: the more negative the fertilization potential amplitude, the greater the susceptibility to polyspermy (Fig. 3). The following results show that the polyspermy is directly attributable to low Na^+ and not to "poisoning" by the Na^+ substitutes used to maintain ionic strength. First, eggs in 120 mM choline $^+$ (360 mM Na^+) are only slightly more polyspermic than controls (Figs. 3 and 4a). Second, eggs are similarly susceptible to polyspermy when Tris $^+$ [Tris(hydroxymethyl)aminomethane], TEA $^+$ (tetraethyl-ammonium), or Mg^{++} are used as impermeant Na^+ substitutes in place of choline $^+$ (Fig. 4a). Li^+ , which can be a permeant substitute for Na^+ (25, and references therein), does not induce polyspermy (Fig. 4a).

If eggs are inseminated in normal seawater, then transferred within 5 min to low Na^+ seawater to reduce the amplitude of the fertilization potential, they will also become very polyspermic (Fig. 4b).

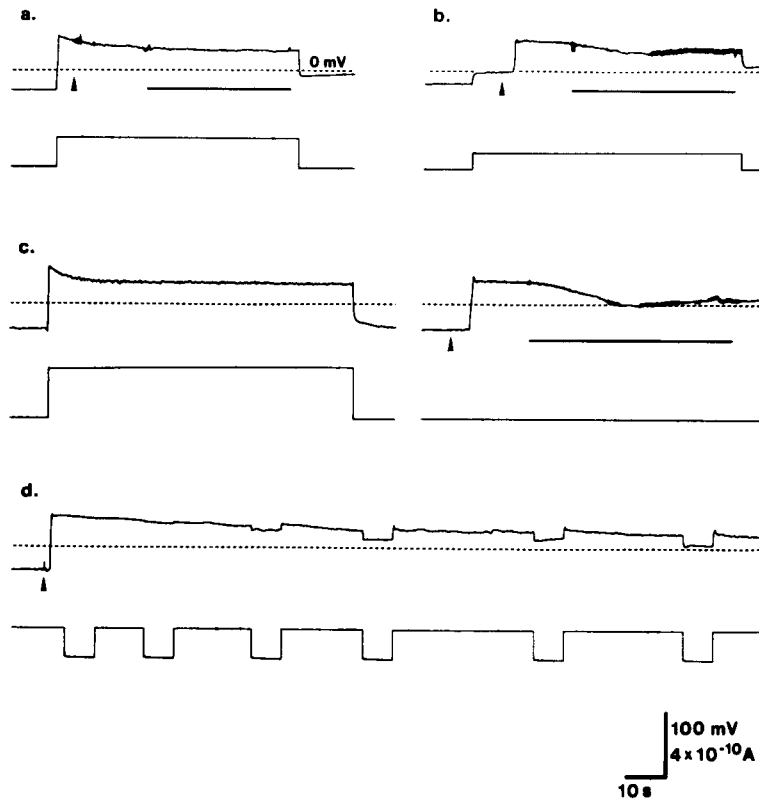


FIGURE 2 Outward and inward current experiments. (a and b) Experiment to test whether positive potential can prevent sperm penetration. The lower trace records current passed into the egg and the upper trace, the egg's membrane potential. Outward current is passed to drive an unfertilized egg's membrane potential to $\geq +25$ mV (a) or to about -5 mV (b), then 5–10 s later sperm are added (arrow). 15–20 s after sperm addition, the dish containing the egg is perfused with seawater in which NaCl is replaced by KCl (496 mM KCl) to inactivate sperm (see Materials and Methods). The high K^+ seawater also activates the eggs, so penetrated sperm nuclei can be scored unambiguously (see Materials and Methods). After ~ 30 s of perfusion (the bar indicates the exact duration) with a total volume of 20–25 ml K^+ -SW, the current is turned off and the egg is allowed to activate, still on the electrode. The penetrated egg and surrounding eggs are then scored for sperm penetration (see Materials and Methods). Neither of the eggs shown in a and b was penetrated by sperm. (c) Control experiment in which outward current is passed before but not during sperm addition. Outward current is passed into an unfertilized egg for at least 60 s (75 s in this case), then current is turned off and sperm are added (arrow) ~ 2 min later. 15–20 s after sperm addition, the dish containing the egg is perfused with K^+ -SW and the eggs are allowed to activate, fixed and scored. The egg in this experiment was penetrated by two sperm. (d) Attempt to bring the fertilization potential to a negative level by passage of inward current. Sperm added at arrow. In this experiment, current pulses of -1.8×10^{-10} A failed to bring the potential below 0 mV during the first 3 min of the fertilization potential.

Less polyspermy is induced by transfer at 10 min, and very little by transfer at 15 min (Fig. 4b). Thus, reducing the membrane potential to more negative values during the fertilization potential results in polyspermy but, after the fertilization potential is over, low Na^+ no longer induces polyspermy.

Reducing External Ca^{++} Has Only a Slight Effect on Polyspermy, and Reducing External K^+ , Mg^{++} , or Cl^- Has No Effect on Polyspermy

Table II shows that reducing external Ca^{++} to 1/10 of its normal concentration (i.e., to 1 mM)

TABLE I
Summary of Outward Current Experiments*

	Minimum potential (mV) during exposure to sperm‡	No. of sperm penetrating
+ Current during sperm addition, $V \geq +25$ mV	+36	0
	+34	0
	+32	0
	+31	0
	+29	1
	+29	0
	+28	1
	+26	1
	+25	0
+ Current before sperm addition, $V \sim -5$ mV	+24	0
	-2	0
	-4	1
	-4	0
	-5	1
	-6	1
	-8	0
+ Current before sperm addition, but not during	-24	1
	-25	1
	-31	1
	-33	3
	-35	1
	-35	1
	-40	2

* Criteria for acceptable experiments: (a) both penetrated and surrounding eggs are activated (nucleolus small or absent; germinal vesicle area indistinct) and well fixed (♀ chromosomes visible and cytoplasm in "good" condition) so that ♂ nuclei would be seen if they were present; (b) the sperm concentration applied was appropriate: $\geq 90\%$ of the surrounding eggs were fertilized (contained sperm) and $\leq 30\%$ of the surrounding eggs were polyspermic (contained more than one sperm).

‡ Diluted $\sim 1:30,000$ ($\sim 10^6$ per ml).

only slightly weakens the polyspermy block. In striking contrast to the 72 sperm per egg in 1/10 external Na^+ (Table II and Fig. 3), heavily inseminated eggs in 1 mM Ca^{++} were penetrated by an average of ~ 5 sperm per egg compared to 2 sperm per egg in 10 mM Ca^{++} . Correspondingly, the average plateau potential after fertilization in 1 mM Ca^{++} is not significantly different from that in 10 mM Ca^{++} (21). However, the maximum amplitude is slightly reduced ($+38 \pm 6$ mV, $n = 12$, in 1 mM Ca^{++} vs. $+51 \pm 6$ mV, $n = 16$, in 10 mM Ca^{++} ; data from reference 21, also compare

Fig. 5 a and b, this paper). The reduced maximum amplitude in 1 mM Ca^{++} is due primarily to the absence of the Ca^{++} action potential at the beginning of the fertilization potential (21). Thus, a slightly reduced maximum fertilization potential amplitude is correlated with a slightly increased susceptibility to polyspermy.

Prolonged exposure to low Ca^{++} causes eggs to become increasingly susceptible to polyspermy (Table II). Therefore, all electrical recordings and tests of the polyspermy block employed eggs preincubated in 1 mM Ca^{++} for 10 min or less before insemination. The increased polyspermy in eggs exposed for a long time to low Ca^{++} may be related to the greater fragility of eggs in low Ca^{++} : they are more easily lysed by stirring or electrode penetration.

Eggs inseminated in 1/10 K^+ (1 mM) or 1/10 Mg^{++} (6 mM) become no more polyspermic than controls (Table II). In fact, they are penetrated by slightly fewer sperm. Factors presumably contributing to the slight decrease in sperm penetration are (a) the tendency for a significant percentage of eggs in some batches to undergo spontaneous activation in the altered solutions (thus establishing a polyspermy block before insemination); and (b) possible adverse effects on sperm, e.g., in low Mg^{++} sperm tend to clump and bind less well to eggs. In 1/7 Cl^- (84 mM), eggs admit the same number of sperm as controls (Table II). Sperm binding appears normal and eggs do not spontaneously activate in low Cl^- . We did not record fertilization potentials in reduced K^+ , Mg^{++} , or Cl^- solutions.

At pH 7, a Transient Polyspermy Block Correlated With a Fertilization Potential Occurs Without Egg Activation

Further support for an electrically-mediated polyspermy block comes from experiments at pH 7, instead of the normal pH 8. At pH 7, sperm enter *Urechis* eggs (37), elicit a fertilization potential and a transient polyspermy block (described below), but the eggs do not activate: the germinal vesicle remains intact and the surface coat does not elevate (37, 39, 50). (Rounding out occurs with the normal time course at pH 7, but the dent reforms at ~ 6 min.) Furthermore, the permanent polyspermy block fails to develop, and reinsemination at pH 7 results in additional sperm entries (references 23, 37, and results below). Thus, at pH 7, the fast polyspermy block and the fertilization

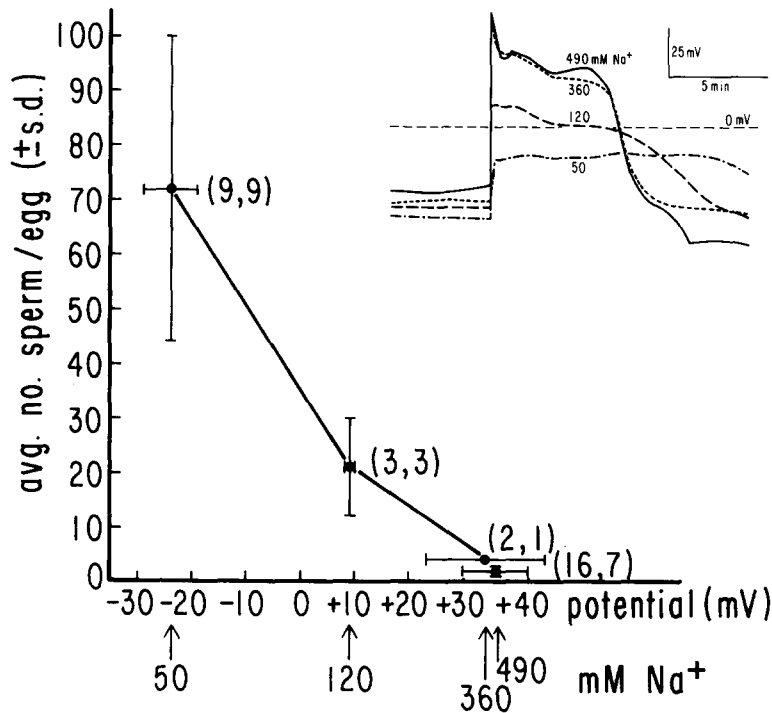


FIGURE 3 Correlation between the average amplitude of the fertilization potential during the first min after insemination and the numbers of sperm penetrating per egg. The amplitude of the fertilization potential was made more negative by decreasing external Na^+ (replacing with choline $^+$); the insert illustrates typical fertilization potentials in normal and reduced Na^+ seawaters. The average amplitude of the fertilization potential during the first min after insemination with a concentrated sperm suspension (1:3,000 dilution) (upper line of the abscissa) was calculated as described in Materials and Methods. The values shown are the averages (\pm SD) of these calculated values from n eggs; this n is the first number in parentheses beside each point in the graph. On the lower line of the abscissa are indicated the corresponding external Na^+ concentrations. The average number of sperm penetrating per egg in these Na^+ concentrations was determined by inseminating a 1:100 egg suspension in a given Na^+ concentration with a concentrated (1:2,400 dilution) sperm suspension. 1 min later, sperm were inactivated with high K^+ -SW, then eggs were fixed and scored for sperm penetration (see Materials and Methods). The values shown are averages (\pm SD) of the average number of sperm per egg from n experiments; this n is the second number in parentheses beside each point in the graph.

potential are correlated, and dissociated from egg activation.

Fig. 6a shows fertilization potentials at pH 7. The amplitude is only slightly reduced compared to that at pH 8; the maximum amplitude is $+41 \pm 5$ mV ($n = 8$), compared to $+51 \pm 6$ mV ($n = 16$) at pH 8 (reference 21); and the average plateau potential (see Materials and Methods) is $+16 \pm 5$ mV ($n = 5$), compared to $+27 \pm 4$ mV ($n = 12$) at pH 8 (21). Reinsemination experiments show that a transient polyspermy block occurs at pH 7. Two experiments are shown in Table III (three additional experiments gave similar results). First, note that these data confirm (see Introduction and Dis-

cussion) that the polyspermy block at pH 8 is not absolute: upon reinsemination, some eggs will admit more sperm. Second, subtracting the number of sperm penetrations resulting from reinsemination at pH 8 from the number at pH 7 ("pH 7-pH 8" in Table III) shows that eggs at pH 7 develop a transient polyspermy block during the first 6 min after insemination. However, by 10 min, their susceptibility to polyspermy has increased, indicating failure to develop the permanent polyspermy block.

At pH 7, few additional sperm enter upon reinsemination at 10 min, probably because under these conditions reinsemination elicits a second

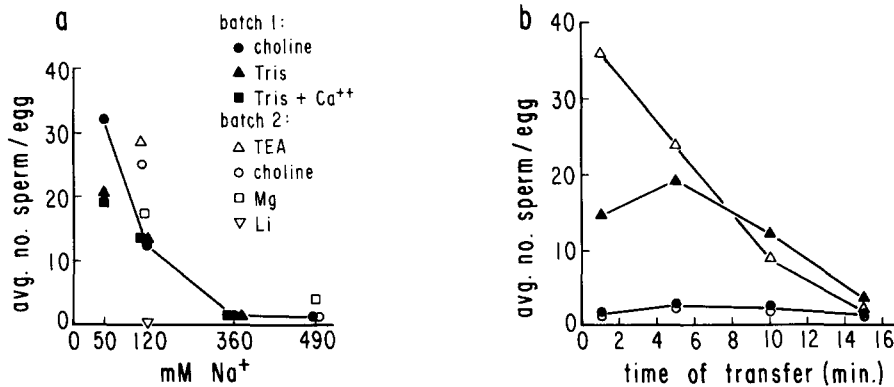


FIGURE 4 (a) The effect of various Na⁺ substitutes on susceptibility to polyspermy. Choline⁺, Tris⁺, (Tris [hydroxymethyl] aminomethane), TEA⁺ (tetraethylammonium), Mg⁺⁺, and Li⁺ were used as partial replacements for Na⁺ in seawater. 490 mM Na⁺ points are controls in artificial seawater with no Na⁺ substituted. Osmolarity of Tris⁺-substituted seawater was adjusted to that of normal seawater by additional Tris⁺. In a control experiment, extra Ca⁺⁺ was added to compensate for reduced Ca⁺⁺ activity in seawater containing large amounts of Tris⁺ (35); polyspermy susceptibility was the same in Tris⁺-SW whether or not extra Ca⁺⁺ was present. Eggs were diluted 1:100 and sperm 1:20,000 in all experiments except for the one with Mg⁺⁺-SW (120 mM Na⁺, 300 mM Mg⁺⁺) and its control; since sperm clumped and did not bind as well to eggs in high Mg⁺⁺-SW, we increased the sperm concentration (1:500 dilution). Filled symbols = egg batch 1; open symbols = egg batch 2. (b) Test for reversibility of the polyspermy block by transfer to 120 mM Na⁺ at various intervals after insemination in normal (490 mM Na⁺) seawater. Eggs were inseminated (1:20,000 sperm dilution), then transferred after 1, 5, 10, or 15 min to 120 mM Na⁺ seawater (triangles) or normal seawater (circles) and immediately reinseminated (1:2,500 sperm dilution). Two experiments shown. Four other experiments gave similar results.

TABLE II
Effect of Ion Substitution on Polyspermy*

Solution	n	Avg. No. Sperm per Egg ± S.D.	Control (normal sea water) Avg. No. Sperm per Egg ± S.D.
1/10 Na (50 mM)	9	72 ± 28	2.0 ± 0.5
1/10 Ca‡ (1 mM)	4	5.2 ± 1.6	2.2 ± 0.5
1/10 Ca§	3	17 ± 6	2.5 ± 0.3
1/10 Mg (6 mM)	3	0.9 ± 0.3	2.8 ± 0.6
1/10 K (1 mM)	4	1.3 ± 0.3	2.6 ± 1.0
1/7 Cl (84 mM)	3	3.3 ± 0.1	2.7 ± 0.7

* Eggs (1:100 dilution) were exposed to sperm (1:2,500 dilution of "dry" sperm in 1/10 Na⁺; 1:500 in the other solutions) until fixation at 30 min.

‡ Preincubated 5 min or less before sperm addition.

§ Preincubated 60 min before sperm addition.

|| number of experiments.

fertilization potential (Fig. 6a) which again establishes a transient polyspermy block.

During the experiment shown in Fig. 6a, seawater was constantly perfused through the recording chamber to wash away excess sperm; an earlier experiment had shown that, without the perfusion,

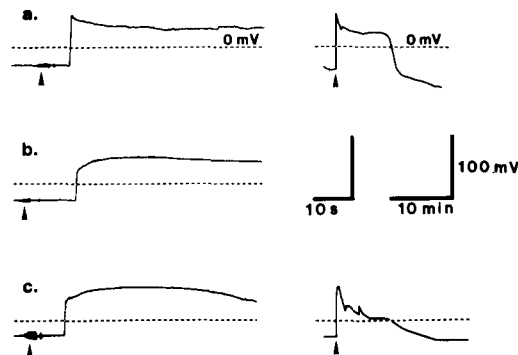


FIGURE 5 Fertilization potentials in normal seawater (a), 1 mM Ca⁺⁺ (b), and normal seawater plus 0.1 mM D600 (c). Arrows mark the time of sperm addition. On the left is a copy of the original chart recording showing approximately the first minute after insemination. On the right is a replot of data on a contracted time scale. Recordings in (a) were from two different eggs; the recording in c was made at pH 7.

the potential stayed positive for over 20 min and was interrupted by a series of step voltage rises, suggestive of a series of overlapping fertilization potentials. Even with perfusion, the duration of

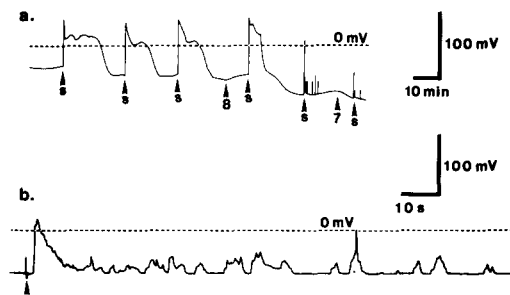


FIGURE 6 Fertilization potentials at pH 7, and reinsemination at pH 7 and 8. (a) An egg perfused with pH 7 seawater was inseminated three times, at $T = 0, 24$ and 44 min (arrows marked "s"), and responded each time with a fertilization potential. The egg (which had not activated) was then perfused with pH 8 seawater (beginning at arrow marked "8") and reinseminated twice more. A fertilization potential was elicited by the first, but not the second, insemination. The seawater was again changed to pH 7, and the egg (now activated) was reinseminated a sixth time: no fertilization potential occurred. (b) Electrical response to reinsemination at pH 8 of an egg (not the one in a) that had been fertilized 15 min earlier at pH 8.

TABLE III
Avg. No. of Sperm per Egg which Penetrated during a 1 min "Pulse" Reinsemination at pH 7 or 8

		Time (min) at end of 1-min pulse reinsemination		
		1.5	6.5	11.5
Exp. 1	pH 7	0.06	0.23	1.66
	pH 8	0.06	0.24	0.58
pH 7-pH 8		0	-0.01	1.08
Exp. 2	pH 7	0.43	0.83	1.73
	pH 8	0.15	0.48	0.10
pH 7-pH 8		0.27	0.35	1.63

Eggs were inseminated at $T = 0$ (1:15,000 sperm dilution); then reinseminated (1:500 sperm dilution) during 1-min pulses (see Materials and Methods) terminating at the times shown. The number of sperm entering during the pulse was calculated by subtracting the average number of sperm in singly inseminated eggs from the average number in doubly inseminated eggs.

fertilization potentials at pH 7 (14 ± 2 min, $n = 4$) is longer than at pH 8 (9 ± 1 min, $n = 10$; reference 21); this may be explained by multiple sperm penetrations if all extra sperm were not washed away before the end of the transient polyspermy block.

Although reinsemination of unactivated eggs at pH 7 causes repeated fertilization potentials, reinsemination of activated eggs at either pH 7 or 8 produces a series of small-amplitude (25 mV maximum), short-duration (1-5 s) responses (Fig. 6). These responses are not caused simply by mechanical jiggling of eggs by swimming sperm, since addition of a very concentrated suspension of sea urchin sperm to an activated *Urechis* egg (at pH 8) caused only two small depolarizations during 5 min, while a comparable insemination with *Urechis* sperm caused 90 responses in 5 min. Thus, activated eggs lose the ability to produce a fertilization potential in response to sperm, despite the fact that sperm are close enough to the egg plasma membrane to elicit a short electrical response. Electron microscopy has shown that acrosomal tubules of supernumerary sperm approach within 100 \AA of the egg plasma membrane, but fusion was never observed (40).

Evidence for an Electrical, Rather than Chemical, Mechanism for the Polyspermy Block

The above results indicate that the early polyspermy block in *Urechis* eggs is mediated by the fertilization potential; the more positive the amplitude of the fertilization potential, the greater is the protection against polyspermy. An obvious question is whether the positive membrane potential per se prevents sperm penetration, as some consequence of a change in electric field across the membrane, or whether sperm penetration is inhibited due to chemical effects of translocated ions. Therefore, we determined the effectiveness of the polyspermy block while varying membrane potential and ion fluxes independently. During the fertilization potential, there are major changes in the fluxes of Na^+ , Ca^{++} , and H^+ (21, 23, 39), but not K^+ and Cl^- .² H^+ efflux has no role in establishing the early polyspermy block since at pH 7 this block develops (Table III), but H^+ efflux is reduced to <5% of that in pH 8 seawater (reference 37, footnote 2). Thus, we considered only Na^+ and Ca^{++} . The experiments described below provide evidence favoring an electrical mechanism: the polyspermy block develops when the membrane potential goes positive, even though Na^+ or Ca^{++} influx is small; and the polyspermy block fails to develop when the membrane poten-

² Authors' unpublished results.

tial remains negative despite large influxes of Ca^{++} and Na^+ .

To determine whether a nearly normal fertilization potential is sufficient to protect eggs against polyspermy when Ca^{++} uptake is small, we reduced the Ca^{++} uptake by inseminating eggs in 1 mM Ca^{++} , or in normal seawater in the presence of the drug D-600. Several other methods for reducing Ca^{++} uptake were unsatisfactory because either Ca^{++} influx was not inhibited very much (Mn^{++}) or there were adverse effects on eggs or sperm (Co^{++} , La^{+++} , Ca^{++} -free seawater).

Fig. 7a shows that Ca^{++} influx during the first minute after insemination in 1 mM Ca^{++} is only 28% of that in 10 mM Ca^{++} . However, the fertilization potential is nearly normal in 1 mM Ca^{++} (reference 21, and see results above), and the polyspermy block at 1 min after insemination is only slightly weakened (eggs tested by reinsemination admitted a significant number [3] of additional sperm in only one of three batches tested).

D-600 blocks Ca^{++} uptake into mammalian cardiac and smooth muscle (26; other references in 27) and inhibits the Ca^{++} action potential of starfish eggs (48), but can also affect movements of other ions (e.g., 27 and references therein). Fig. 7b shows that Ca^{++} influx during the first minute after insemination in the presence of 0.1 mM D-600 is reduced to only 10% of control values. The effects of D-600 on electrical properties of *Urechis* eggs are similar to those of reduced external Ca^{++}

(Fig. 5). The drug eliminated the Ca^{++} action potential (in three of three unfertilized eggs tested) and increased the time for the fertilization potential to reach maximum amplitude, from 1 s (reference 21) to 15 and 60 s (in two eggs tested). However, the average amplitudes during the first minute after insemination were +38 and +40 mV, comparable to that (+36 mV) in controls. Like 1 mM Ca^{++} , D-600 only slightly weakens the polyspermy block. Eggs in 0.1 mM D-600 inseminated with a 1-min pulse of sperm (1:500) admitted an average of 3.9 ± 1.3 sperm per egg compared to 1.8 ± 0.4 sperm per egg in controls (two expts.). Therefore, Ca^{++} influx can be substantially reduced under conditions in which the fertilization potential is nearly normal, and the polyspermy block is only slightly weakened.

A similar conclusion can be drawn with respect to Na^+ influx. Fig. 2 and Table II show that positive potential, produced by application of outward current, is sufficient to inhibit sperm entry. Since the current pulse causes no conductance increase comparable to that occurring during the fertilization potential, we infer that there is no comparable Na^+ influx. This argues that Na^+ influx is not necessary to establish the polyspermy block.

Results of reciprocal experiments, i.e., keeping the membrane potential negative while permitting large influxes of Na^+ and Ca^{++} , also suggest that the membrane potential, rather than specific ion

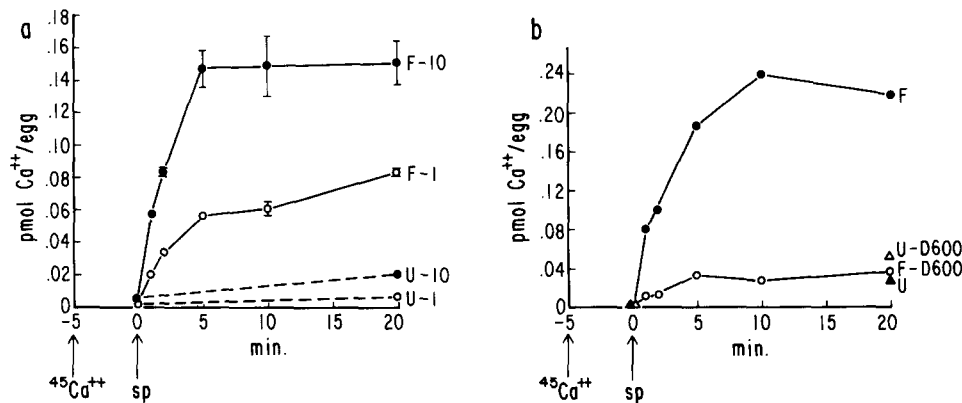


FIGURE 7 Uptake of Ca^{++} in seawater containing 1 mM Ca^{++} (a) or 0.1 mM D600 (b). $^{45}\text{Ca}^{++}$ (final concentration $4 \mu\text{Ci/ml}$) was added to eggs (1:50) in the test solution or in normal seawater for controls. 5 min later, an aliquot was removed for radioactivity determination and sperm (1:10,000 dilution in a; 1:5,000 in b) were added immediately thereafter to the rest of the eggs (except for unfertilized samples). Additional aliquots were removed at the times indicated and processed as described in Materials and Methods. Points in (a) are averages of two experiments ($\pm\text{SD}$); brackets are omitted where $\text{SD} \leq$ diameter of the point. F, fertilized; U, unfertilized; 10, normal seawater; 1, 1 mM Ca^{++} seawater.

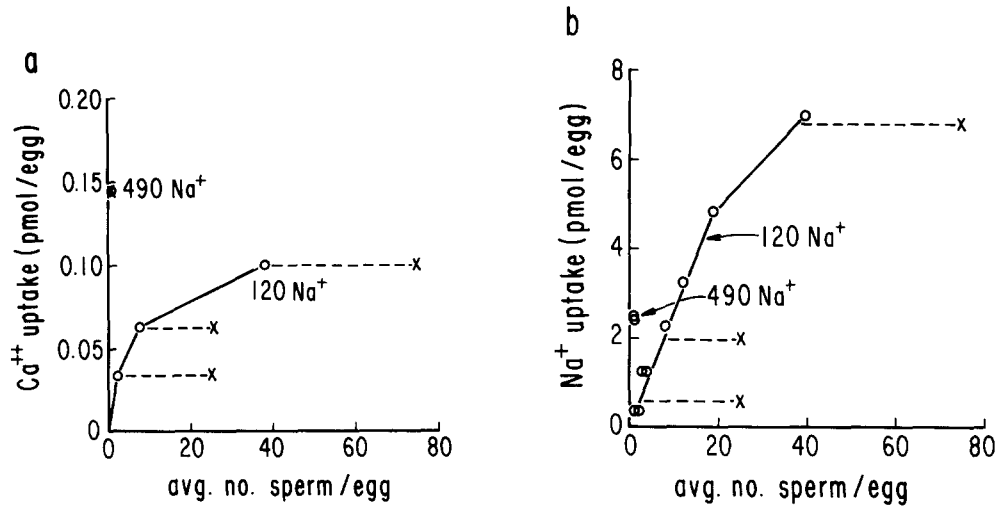


FIGURE 8 (a) Ca^{++} uptake and polyspermy susceptibility in 120 mM Na^+ . Eggs (1:50) were added to $^{45}\text{Ca}^{++}$ (final concentration 2 $\mu\text{Ci}/\text{ml}$) plus varying amounts of sperm (diluted 1:100,000 to 1:2,500) in 490 or 120 mM Na^+ -SW. At 5 min after insemination, one aliquot was removed for measuring tracer uptake, a second aliquot was removed to score for numbers of sperm penetrating during the first 5 min (sperm inactivated with K^+ -SW; see Materials and Methods), and a third aliquot was reinseminated (5-min pulse of sperm at 1:2,500 dilution) to test for a polyspermy block. Circles denote the average number of sperm penetrating per egg during the initial 5-min insemination; X's denote the average number of sperm penetrating per egg after insemination plus reinsemination. (b) Na^+ uptake and polyspermy susceptibility in 120 mM Na^+ . At $T = 0$, eggs (1:50) were added to $^{24}\text{Na}^+$ (final concentration 7.5 $\mu\text{Ci}/\text{ml}$) plus varying amounts of sperm (dilution 1:100,000 to 1:2,500) in 490 or 120 mM Na^+ -SW. At 5 min after insemination, one aliquot was removed to measure tracer uptake, and a second aliquot was removed to score for sperm penetration. The Na^+ influx data (circles) were obtained from two egg batches and are replotted from reference 21. The reinsemination data (X's) were obtained from the experiment in a.

influxes, protects the egg against polyspermy. The design of these experiments takes advantage of our finding (reference 21; Fig. 8, and see Introduction) that eggs inseminated in 50 or 120 mM Na^+ take up Na^+ and Ca^{++} in proportion to the number of sperm which enter them, but because of the low external Na^+ the fertilization potential amplitude is reduced. Thus, we "injected" varying amounts of Na^+ and Ca^{++} by varying sperm concentration and then determined whether the eggs established a polyspermy block. Fig. 8 compares susceptibility to polyspermy in 120 mM Na^+ seawater, with Ca^{++} and Na^+ influxes during the first 5 min after insemination. Eggs in 120 mM Na^+ were highly susceptible to polyspermy despite Ca^{++} influxes of up to 70% of control values in normal seawater (Fig. 8a) and despite Na^+ influxes even greater than in normal seawater (Fig. 8b). Reinsemination of these eggs at 5 min indicated that they remained highly susceptible to polyspermy and that there was no correlation between the number of sperm entering upon reinsemination and the amount of Ca^{++} or Na^+ uptake that had occurred before that

time. A second experiment (not shown) gave essentially the same results as those shown in Fig. 8a and b.

In summary, under experimental conditions of almost normal Ca^{++} influx and even greater than normal Na^+ influx, *Urechis* eggs remain highly susceptible to polyspermy if the amplitude of the fertilization potential is reduced. Conversely, under conditions in which there is little Na^+ or Ca^{++} influx, sperm entry is inhibited if the potential is sufficiently positive.

DISCUSSION

The fertilization potential in *Urechis* eggs is a positive shift in membrane potential accompanied by an influx of Na^+ and Ca^{++} , which is elicited by fertilization and lasts 5–10 min thereafter (21). The results show that the fertilization potential in *Urechis* functions as a block to polyspermy, preventing supernumerary sperm from entering the egg. In addition, our results indicate that the block is a direct result of the positive membrane potential rather than a secondary effect of the translocated

ions. These findings raise several issues which we will discuss below: the nature of the relationship between membrane potential and susceptibility to polyspermy, the generality of this strategy for polyspermy prevention, and, finally, its molecular mechanism.

Potential Dependence of the Probability of Fertilization

During the early, electrically-mediated phase of the polyspermy block in both *Urechis* and sea urchin eggs, the probability of a second sperm entry is greatly reduced but is not zero. In heavily inseminated eggs of both animals, a second sperm can enter during either the rise phase of the fertilization potential or the positive plateau (38, 20, 46, and our results). Furthermore, when the potential of unfertilized *Urechis* eggs is held at a level comparable to that during the fertilization potential, sperm penetration sometimes occurs (Table I). In similar experiments with sea urchin eggs (20), sperm penetration never occurred; however, in these experiments (20) the eggs were not challenged with higher sperm concentrations, and the data of Rothschild and Swann (46) show that the block can fail during the period corresponding to the fertilization potential plateau, if sperm-egg ratios are high enough. Thus, we conclude that in both *Urechis* and the sea urchin the positive potential does not absolutely exclude sperm. A lag in propagation of the potential change around the egg surface cannot explain the "leakiness" of the electrical polyspermy block: because the resistance through the egg interior is so small relative to that across the membrane, eggs are isopotential systems, unlike nerve and muscle. Although the polyspermy block is not absolute, it will be effective if the probability of sperm entry is sufficiently reduced.

Generality

In sea urchins, the fertilization potential provides an electrical polyspermy block that protects the eggs during approximately the first minute after insemination, before the permanent cortical vesicle-mediated block is established (20). In contrast, the electrical block in *Urechis* protects the egg for 5–10 min (above results) and is not superseded by a cortical vesicle mediated block but by a plasma membrane block of unknown mechanism (40).

The eggs of anuran amphibians also produce a

positive-going fertilization potential (7, 19, 31), and recent electrophysiological experiments indicate that this response provides an electrical polyspermy block (7). Passing current to hold the membrane potential of the unfertilized egg positive during insemination delays fertilization until the current is turned off, while using ion-substituted media to reduce the amplitude of the fertilization potential to more negative values promotes polyspermy. Polyspermy can also be induced by fertilizing in a solution with a high concentration of Cl^- ions (2, 15), which probably reduces the amplitude of the fertilization potential (19, 31). Thus, it can be concluded that the anuran amphibian egg has an electrical polyspermy block; this may explain the observation that a polyspermy block is observed even after removal of the vitelline envelope (24).

In the fish *Oryzias*, the most positive level reached during the fertilization potential is only about -20 mV (34). Voltage clamp experiments show that sperm entry was not inhibited by membrane potentials between -80 and $+50$ mV during insemination,³ and hence it was concluded that these eggs lack an electrical polyspermy block. Unlike that in the three groups of animals discussed above, sperm access to the *Oryzias* egg is limited to a channel, the micropyle, which is so narrow near the egg surface that sperm are in single file. Within 5–12 s after the sperm enters the egg, the micropyle is plugged by local vesicle secretion (11, 47).

In summary, an electrical polyspermy block occurs in three of four groups of animals investigated. This strategy for polyspermy prevention may be rather common, since fertilization potentials have been observed in eggs of molluscs (10), tunicates (8), annelids,⁴ and hemichordates⁴ (for a review, see reference 16). Also, there is evidence for a polyspermy block at the level of the egg plasma membrane in a variety of organisms. For example, tunicate eggs lack cortical vesicles (45), and in the molluscs *Spisula* (44) and *Mytilus* (18) the cortical vesicles do not undergo exocytosis in response to insemination. In *Spisula* (53) and mouse (52) eggs, a polyspermy block has been demonstrated after removal of the extracellular coats, while in eggs of crinoids (17), rabbits, mice, and rats (3) supernumerary sperm are observed to

³ Nuccitelli, R., University of California, Davis. Personal communication.

⁴ Jaffe, L. A. Unpublished results.

have penetrated the surface coats but not the egg. It will be interesting to learn whether these animals employ an electrical polyspermy block.

Mechanism

Experiments described above show that the susceptibility of *Urechis* eggs to polyspermy during the first 5 min after insemination is correlated with the amplitude of the fertilization potential but not with the magnitudes of Na^+ and Ca^{++} influx or H^+ efflux. Assuming that changes in the magnitudes of these fluxes would produce corresponding changes in cytoplasmic ion activities, our results indicate that changes in the intracellular activities of these ions are not responsible for the polyspermy block. Likewise, since fertilization will occur at pH 7 and in low Na^+ and Ca^{++} solutions, a possible increase in H^+ or decrease in Na^+ or Ca^{++} near the external surface of the plasma membrane during the fertilization potential cannot be responsible either.

In summary, our results indicate that the change in electric field across the membrane, rather than changes in internal or external ion activities, causes the electrical polyspermy block. This block apparently acts at the level of sperm-egg membrane fusion (see Introduction and reference 40). We know of no other studies, besides those demonstrating electrical polyspermy blocks, which demonstrate that membrane potential per se can regulate membrane fusion.

It can be argued that the potential-sensitive element responsible for the electrical polyspermy block should be in the plasma membrane: since the electrical resistance of the membrane is much higher than that of the surrounding salt solutions, essentially all of the potential drop will occur across the membrane. Direct effects of the field on cortical structural proteins such as microfilaments and microtubules are therefore unlikely. Indeed, the finding that cytochalasin B inhibits the plasma membrane polyspermy block in *Spisula*, which was the basis for suggesting that the block was mediated by cortical microfilaments (53), has recently been disputed (reference 30; see also references 4, 14).

We can suggest at least three possibilities for the mechanism of the electrical polyspermy block. The electric field across the egg plasma membrane could regulate sperm-egg fusion by (a) an effect on the fluidity of the lipid bilayer, or (b) an effect on macromolecules embedded in the bilayer (e.g., putative "sperm receptors"), or (c) an electrostatic

repulsion of some charged element in the sperm membrane which must move within the bilayer of the egg membrane to effect fusion. This third hypothesis will not be discussed further, since we know too little about the precise sequence of molecular rearrangements that occur during membrane fusion.

The ability of liposomes to fuse cells depends on the fluidity of the lipids in the liposome (36), and the observation of voltage-dependent phase separation in lipid bilayers (33) suggests that electrical potential might alter membrane fluidity. Hence, a decrease in membrane fluidity might possibly account for the potential-dependent block of sperm egg fusion. Membrane fluidity of sea urchin eggs before and after fertilization has been measured with a spin-labeled fatty acid (5). Fluidity was reported to decrease after fertilization, but the measurements were of bulk, rather than of plasma, membrane fluidity, and omitted the first 10 min after insemination. In a study with mouse eggs (22), lateral mobility of a lipid-soluble fluorescent dye and of Fab fragments was reported to increase after fertilization, but the measurements were made 3 h after insemination.

Alternatively, a change in electric field could prevent fusion by changing the conformation, position, or state of aggregation of fusion-associated proteins, glycoproteins, or glycolipids in the egg plasma membrane, by analogy with the potential-dependent properties of membrane macromolecules such as some ion channels (reviewed in reference 9), lipid-soluble dyes and photosynthetic chromatophores (reviewed in reference 6), and acetylcholine receptors (28). That specific macromolecules in the membrane may be necessary for fusion is supported by freeze-fracture views of intramembrane particles near sites of exocytosis in various cells (references in 49), gametic fusion in *Chlamydomonas* (51), and myoblast fusion during myogenesis (43). It is not known, however, whether fusion occurs at the location of the particles or in adjacent regions devoid of particles (49). Several freeze-fracture studies of eggs and sperm during the fertilization period have been reported (29, 32, 41, 42), but whether the observed particles are involved in sperm-egg fusion is unknown.

Although the studies cited above do not show how positive membrane potential inhibits sperm-egg fusion, they do suggest the feasibility of a number of approaches to this question. For example, it would be interesting to determine whether changes in membrane properties, such as

fluidity, or the conformation and/or distribution of membrane constituents are correlated with the establishment of the electrical polyspermy block. *Urechis* appears to be favorable material for pursuing the question of mechanism by virtue of the long duration of the electrical block, the absence of cortical vesicle exocytosis, and the facility with which potential and ion fluxes can be varied independently. Furthermore, the probability of sperm-egg fusion can be controlled predictably. We hope that further studies of the mechanism of the electrical polyspermy block will not only increase our understanding of the biology of fertilization but may also contribute to an understanding of the necessary and sufficient conditions for membrane fusion in general.

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