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Time and voltage windows for reversing the electrical block to fertilization

(membrane fusion/membrane potential/activation/facilitation/sperm)

SHELDON S. SHEN* AND RICHARD A. STEINHARDT†‡

*Department of Zoology, Iowa State University, Ames, IA 50011; and †Department of Zoology, University of California, Berkeley, CA 94720

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ABSTRACT **The electrical block to fertilization of sea urchin eggs can be overcome by very brief periods of inside-negative egg membrane potential. *Lytechinus pictus* eggs whose membrane potentials have been clamped at +15 mV cannot be fertilized. If the membrane potential is repolarized to inside-negative voltages for a brief interval, the egg can be successfully fertilized. By varying the duration and voltage of these brief periods of inside negativity, we have uncovered three general properties of the electrically sensitive step in fertilization. First, a membrane-potential step that becomes rate limiting at inside-positive voltages can be initiated within a few milliseconds of inside negativity (30–60 msec at −60 mV). Second, at the time that the electrically sensitive step is being completed, there are other potential-independent steps with probably slower time constants because the duration of negativity was more effective applied as paired pulses rather than a single long pulse. Third, the permissive state is more quickly established by inside negativity than the nonpermissive state is established by inside positivity because the interval between paired pulses could be a few times longer than the effective single pulse in duration. In these voltage-clamped eggs the intervals from the successful completion of the electrically sensitive step to the next identifiable signs of activation were on the order of several seconds and highly variable.**

At fertilization in a sea urchin egg, the first successful sperm triggers rapid permeability increases to calcium and sodium that reverse the polarity of the egg membrane potential to inside-positive values (1–4). This rapid depolarization to inside-positive membrane potentials prevents fertilization by secondary sperm (1). This is called the electrical block. These permeability changes do not appear to play a direct role in the activation of development (5). However, the inside-positive values of membrane potential that result from fertilization by the first sperm can serve as the basis for the fast block to polyspermy in the eggs of sea urchins (1), the marine worm *Urechis* (6), and the frog *Xenopus* (7). The electrical block can also be artificially imposed by the application of current through an intracellular electrode so that the inside-positive membrane potentials prevent any sperm from completing the fusion and activation process (1).

Although there is no direct evidence on how the positive inside values of egg membrane potential block the completion of fusion of the sperm with the egg and the activation of the egg, there is suggestive evidence that the voltage-sensitive component is a property of the sperm and not the egg membrane (8, 9). This implies that the electrical block is directly interfering with the fusion process itself, because the voltage-sensitive component of the sperm can be influenced by the egg membrane potential only when it is inserted at least partially across the egg membrane resistance. In the experiments described here, we have attempted to further characterize the steps leading to the completion of the fusion process and activation of the egg by momentarily reversing the electrical block with short pulses of inside negativity.

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MATERIALS AND METHODS

Gametes and Solutions. Eggs and sperm of the sea urchin *Lytechinus pictus* were obtained by injection of 0.5 M KCl into the coelomic cavity. The sperm were collected dry and stored at 4°C. The jelly coats were removed from the eggs by passing them through fine mesh silk and then the eggs were washed twice in artificial sea water (ASW). The dejellied eggs were maintained at 16–18°C and constantly stirred at 60 rpm. All experiments used eggs within 4 hr of shedding and were done in ASW of the following composition: NaCl, 470 mM; KCl, 10 mM; $CaCl_2$, 11 mM; $MgSO_4$, 29 mM; $MgCl_2$, 27 mM; $NaHCO_3$, 2.5 mM; pH 8. The spermicide uranyl nitrate was used at 0.3 mM in ASW (pH 6.3). This solution was prepared by adding stock solution of 10 mM uranyl nitrate in distilled water to an appropriate amount of ASW (10).

Electrophysiology. Eggs were held on poly(lysine)-coated plastic Petri dishes (Falcon 1008). Voltage clamp experiments were performed by the conventional two-intracellular-microelectrodes technique with the Biodyne AM-5 voltage clamp system (Biodyne Electronics, Santa Monica, CA). The microelectrodes were pulled from 1.2-mm omega-dot tubing, filled directly with 3 M KCl, and had resistances of 40–80 MΩ. The membrane potential was recorded as the potential difference between the intracellular potential microelectrode and a third KCl-filled microelectrode in the bath. A 3% agar–ASW bridge between bath and 3 M KCl/Ag–AgCl to ground served as the indifferent electrode. The applied current through the intracellular current electrode was monitored with a Biodye CV-1 current–voltage converter inserted between the indifferent electrode and ground. The command pulse was delivered by a W-P Instruments model 301 stimulator (New Haven, CT) with a model 305 isolation unit. The applied command pulse was monitored on a Tektronix 5111 oscilloscope (Beaverton, OR) and permanent records of voltage and current were made with a Soltec model 3314 chart recorder.

The following experimental protocol was used. An egg was impaled with two microelectrodes by negative capacitance. After stabilization of the membrane potential, the egg was voltage clamped to an inside positive value. The voltage clamp was set so that a stimulus pulse delivered a command voltage of specific amplitude and duration or, in some cases, a pair of pulses within a specific interval. Approximately 0.1 ml of freshly diluted sperm at 10^7 sperm per ml was placed 3–5 mm from the egg. Fertilization of surrounding eggs was scored by elevation of the fertilization envelope. Fertilization of the voltage-clamped egg was scored by both the change in applied current due to the change in the membrane

Abbreviation: ASW, artificial sea water.

‡To whom reprint requests should be addressed.

resistance (11) and the subsequent elevation of the fertilization envelope. A change in clamp current was observed only in eggs that then elevated their fertilization envelope. Eggs that elevated fertilization envelopes subsequently divided. When >90% of the neighboring eggs had elevated their fertilization envelope and the voltage-clamped egg had not, the predetermined command pulse was applied. If fertilization did not ensue, a second command pulse of longer duration was applied. No more than four command pulses were tested on any one voltage-clamped egg. In some experiments, additional fresh sperm were added to the vicinity of the egg prior to a command pulse. After monitoring fertilization, the voltage clamp was removed and the microelectrodes were withdrawn from the egg. Experiments with greater than ±3 mV drift were discarded.

Uranyl Nitrate. Experiments with the spermicide, uranyl nitrate, were performed as described by Presley and Baker (10). A 2% suspension of eggs was mixed with an equal volume of ASW containing 2×10^7 sperm per ml and constantly agitated. At the desired time intervals, 1-ml samples of the sperm/egg mixture were transferred to 30 ml of 0.3 mM uranyl nitrate in ASW and kept gently agitated. In all experiments, zero time was taken as the time of addition of eggs to the sperm mixture and duration of fertilization was taken as the time up to decanting into spermicide. At 10 min after fertilization, the eggs were fixed in 100% ethanol/acetic acid (3:1) for 24 hr, then cleared in 60% acetic acid and scored for the presence of sperm pronuclei. A minimum of 50 eggs in each sample was scored and the data from two egg donors were pooled.

RESULTS

Determination of Blocking Voltage. The electrical block to fertilization is not absolute (8). However, it is possible to adjust the membrane potential to a high enough inside-positive value so that at a given sperm concentration, fertilization is a highly improbable event (5). An initial set of experiments was performed to determine the inside-positive clamp voltage that would block fertilization at the sperm concentration used in our experiments. The clamp data for various inside-positive egg membrane potentials and the number of successful fertilizations at each potential are given in Table 1. From these results, it was determined that for *L. pictus* it is necessary to set the egg membrane potential at +15 mV to block fertilization under the conditions of our experiments. In all the following experiments, we found that a clamp voltage of +14 to +16 mV inside positive was sufficient to block fertilization when 0.1 ml of sperm suspension (10^7 per ml) was added 3–5 mm from the egg.

Time and Voltage Windows for Reversing the Electrical Block. In these experiments, we determined the duration of negative egg membrane potential required to overcome the electrical block to fertilization. The basic protocol for these experiments is illustrated in Fig. 1. An egg was clamped at +15 mV and sperm were added to the dish. In less than a minute, the *surrounding unclamped* eggs had fertilization envelopes, indicating successful fertilization. The clamped egg remained unfertilized, showing no activation current and no

Table 1. Fertilization block by voltage clamp

Clamp voltage, mV	Trials, no.	Fertilized, no.
+22 ± 1	2	0
+20 ± 1	6	0
+15 ± 1	73	1
+10 to +13	12	9
0 to +9	6	6

Fertilization criteria were change in clamp current and start of elevation of the fertilization envelope by 90 sec after addition of sperm.

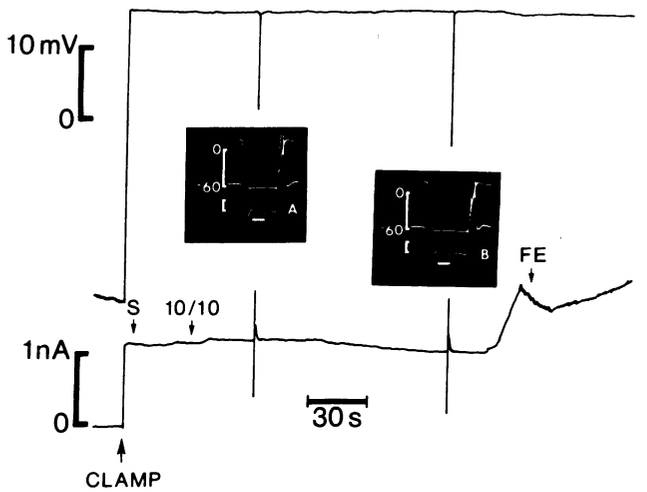

FIG. 1. Chart recording of egg membrane voltage and clamp current during time and voltage window for reversal of the electric block to fertilization. An egg with resting potential of −23 mV (after penetration with voltage and current microelectrodes) was clamped to +15 mV with a 1.1-nA outward current. At "S" 0.1 ml of freshly diluted sperm suspension (10^7/ml) was added 4 mm from the clamped egg. By 30 sec, all 10 neighboring eggs had started to elevate fertilization envelopes, while the voltage-clamped egg remained unchanged. However, after an inward command pulse of 36-msec duration, which hyperpolarized the egg to −60 mV (oscilloscope record, *Inset B*), the egg fertilized. Fertilization was characterized by an increase in outward current 18 sec later (at the clamped egg potential of +15 mV) followed by elevation of the fertilization envelope (FE). An earlier command pulse of 30-msec duration (*Inset A*) was inadequate for fertilization to ensue. For both *Insets A* and *B*, the calibration bars are top vertical, 60-mV membrane potential; bottom vertical, 1-nA applied command current; horizontal, 10 msec.

fertilization envelope. After a further delay, a command voltage of −60 mV was imposed for 30 msec (Fig. 1, *Inset A*). The egg was monitored for >90 sec and fertilization did not occur. Another command voltage of −60 mV was imposed, this time for a duration of 36 msec (Fig. 1, *Inset B*). This time the activation current and elevation of the fertilization envelope followed the period of inside negativity in <30 sec. Table 2 summarizes 115 trials of this type in which we judged the effectiveness of a single period of given duration and voltage on reversing the electrical block.

Two main conclusions were clear from the data in Table 2. First, it was possible to overcome the electrical block with a very short period of inside-negative egg membrane potential.

Table 2. Time and voltage window for fertilization

Command voltage, mV	Window duration, msec	Trials, no.	Fertilized, no.
−15	30–40	8	1
−15	41–50	8	2
−15	51–60	7	2
−15	61–70	7	1
−15	71–80	8	2
−60	15–19	2	0
−60	20	34	0
−60	25–30	17	2
−60	36–40	14	9
−60	43–70	10	9
−90	10–15	5	0
−90	16–20	9	3
−90	22–30	10	6
−90	35–40	6	6

Blocking clamp voltage, +15 ± 1 mV.

As little as 28 msec at −60 mV could result in successful fertilization, approaching an almost certain success with 40 msec at −60mV. Second, the probability of successful fertilization increased with both duration and voltage, at least within the limits tested here. With a command voltage of −60 mV, only 2 out of 17 trials were successful with durations of 21–30 msec. At command voltages of −90 mV, 6 out of 10 trials succeeded with durations of 21–30 msec. At −15 mV, even the longest durations tested (71–80 msec) had a low proportion of successes.

Facilitation of Fertilization with Paired Pulses. The probability of successful fertilization was higher when the periods of inside negativity were imposed as two brief paired pulses separated by an interval in the millisecond range in which the egg membrane potential was returned to the +15 mV blocking potential (Fig. 2). When applied in this manner, periods of inside negativity are much more effective at permitting fertilization (Table 3). A total period of 20 msec was never successful (34 trials) at a command voltage of −60 mV when applied as a single pulse. However, the same total time applied as paired separate 10-msec pulses resulted in a high rate of successful fertilization (9/23 trials at intervals between pulses of <60 msec). The longest interval between 10-msec pulses (to −60 mV) in a successful fertilization was 55 msec; between 20-msec pulses the longest intervals in successful trials were 80 msec long.

The Electrically Sensitive Step in Relation to the Commitment to Activate. The time and voltage windows also provide information on the timing of subsequent steps of the activation process. The normal sequence on fertilization begins with attachment of the successful sperm followed closely by the depolarization it induces. This first successful sperm must have passed the electrically sensitive step and committed the egg to activate before the rapid depolarization establishes the electrical block. Since the initial rapid depolarization has been seen within 3 sec (1, 2), it is evident that the process of activation can be set in motion within seconds.

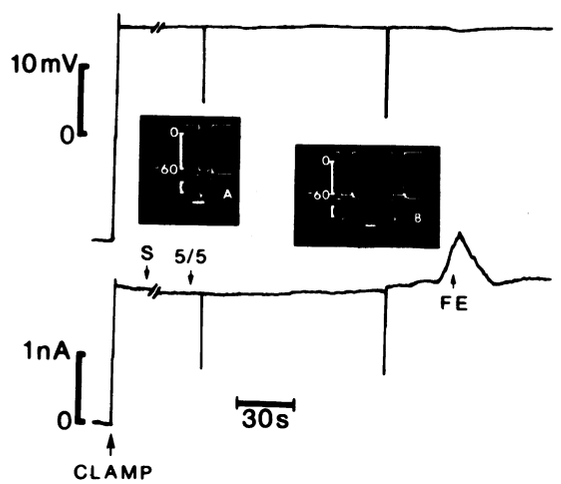

FIG. 2. Facilitation of time and voltage window for fertilization. An egg was clamped at +15 mV by a 1.9-nA outward current. At "S", sperm were added and by 2 min (as shown by the break in the record), all five neighboring eggs had started to elevate fertilization envelopes. In all facilitation experiments, a single pulse was tested first without overcoming the electric block. In this experiment, a single command pulse 20 msec long (*Inset A*) was not followed by fertilization. However, two 20-msec pulses delivered 70 msec apart (*Inset B*) was followed 15 sec later by the increase in the clamp current associated with the permeability changes of a successful fertilization and elevation of the fertilization envelope. For both *Insets A* and *B*, the calibration bars are top vertical, 60-mV membrane potential; bottom vertical, 2-nA applied command current; horizontal, 20 msec.

Table 3. Facilitation of time and voltage windows for fertilization by pairing pulses

Window, duration, msec	Interval duration, msec	Trials, no.	Fertilized, no.
20	95–150	8	0
20	80–90	9	2
20	60–75	7	5
10	50–90	13	1
10	35–48	7	3
10	9–32	11	5

Blocking clamp voltage, +15 ± 1 mV. Command voltage, −60 mV.

In these experiments, the time and voltage windows show that only tens of *milliseconds* of inside negativity are required to get past the electrically sensitive step and commit the egg to activate. Under our conditions, the voltage-clamped egg does not show the early electrical events such as the permeability changes associated with the calcium action potential because these channels are inactivated by the inside-positive voltages. Similarly, the large currents associated with the sodium-dependent depolarization are attenuated because the membrane potential is close to the equilibrium potential for sodium. The first sign of activation after a successful window of negativity is the large outward current reflecting changes in membrane permeability just before the cortical reaction results visually in the elevation of the fertilization envelope. We have called this current the "activation current" for convenience as it is the first sign of a successful activation under our conditions and is always closely associated with onset of the cortical reaction. There is always a relatively long period from the time of a successful window of negativity to the start of the activation current, usually on the order of several seconds (Figs. 1 and 2).

The fact that the already attached sperm overcome the electrically sensitive step at a precise moment allows us to determine the exact intervals to subsequent activation events. In Fig. 3, the percentage of voltage-clamped eggs in our studies that had started the activation current is plotted against time measured from the application of the window of negativity. Fifty percent had started the activation current by 17 sec. The surprising finding is that this interval is highly variable, because each egg starts out with release at the electrically sensitive step. The timing of the activation current is closely similar to the period of irreversible commitment to activation as judged by the block that can be induced by uranyl ions. Uranyl ions applied to sperm and egg suspensions result in 50% inhibition at 22 sec after sperm addition (Fig. 4). These observations, taken together, suggest that the elec-

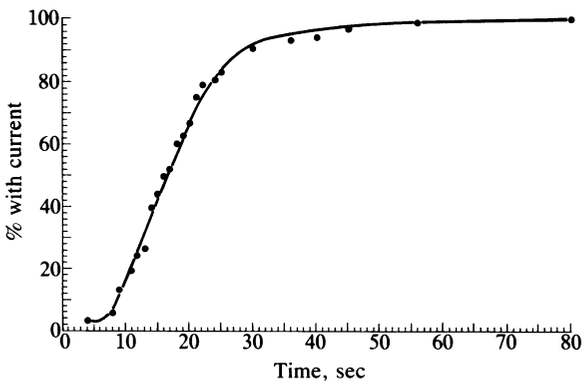

FIG. 3. Plot of percentage of eggs that have started the activation current associated with the permeability changes at a successful fertilization against time from application of the successful pulse of inside negativity.

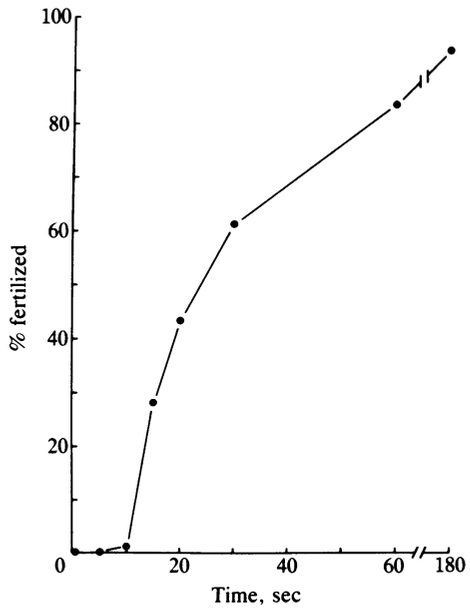

FIG. 4. Fertilization rate curve for uranyl nitrate treatment. Sperm were added to eggs at zero time. At subsequent time intervals, 1-ml samples of the egg/sperm mixture were transferred to 30 ml of 0.3 mM $UO_2(NO_3)_2$ in ASW (pH 6.3). Ten minutes after fertilization, all egg samples were fixed and scored for fertilization.

trically sensitive step is separated by a long and highly variable interval from the subsequent identified events associated with activation.

Low Incidence of Polyspermy After Release of the Electrical Block. Finally, we were somewhat surprised to observe a low rate of polyspermy after the application of a window of negativity. Despite the presence of numerous sperm, all presumedly blocked at the same point, nearly all fertilizations were monospermic, as judged by the elevation of the fertilization envelope from a single point. In 18 cases followed to first cleavage just to check this observation, only one egg failed to divide properly, and that one had been judged polyspermic because it had two sites where the fertilization envelope started to elevate. Under our conditions, 2–3 dozen sperm are attached to the egg at the time of release from the electrically sensitive step.

DISCUSSION

It is unlikely that the electrical block simply reflects the electrocution of sperm as they start to fuse because the extremely short time required to overcome the electrically sensitive step would not allow time for another sperm to attach and reach the prefusion state. Since the voltage required to block fertilization depends on the species of sperm, the best model for the electrical block is a fusion or transfer process in which some component of the sperm is physically inserted into the egg membrane so that it is sufficiently across the resistance of the egg membrane to experience the membrane potential (8, 9). We have used this model in discussing our results on overcoming the electrical block.

The time- and voltage-window experiments reveal three general properties of the electrically sensitive step in fertilization. First, a membrane-potential step becomes rate limiting at inside-positive voltages but is initiated within 30–60 msec at −60 mV. Second, at the time that the electrically sensitive step is being completed, there are other potential-independent steps with probably slower time constants because a given duration of negativity was more effective applied as paired rather than as a single long pulse. Third, the permissive state is more quickly established by inside negativity than the nonpermissive state is established by inside positivity because the interval between paired pulses could be a few times longer than the effective single pulse in duration.

The relatively long highly variable period between the reversal of the electrical block and the start of the activation current is very interesting to us. The precise timing of these reversal experiments reinforces the idea that the bulk of the latency period in fertilization follows the successful completion of the first steps of the insertion/transfer function of the fertilizing sperm. A similar highly variable latency period has been reported in studies of the flagellar motility of the fertilizing sperm. Epel *et al.* (12) have shown that just before the cortical reaction the fertilizing sperm suddenly ceases motility and is incorporated into the egg. The interval between time of attachment and the time when movement ceases ranged between 13 and 25 sec with an average of 19 sec. Our measurements of intervals to the activation current and loss of sensitivity to uranyl ions fall roughly in the same range and also coincide with the time of the cortical reaction. We do not know what follows the electrically sensitive step nor why there is the relatively long and highly variable latency period from when the sperm passes the electrically sensitive step to all the subsequent events of activation. The time- and voltage-window experiments have made clear to us just how little is known about this early portion of the multistep process called simply fertilization.

When the electrical block is released momentarily there is a low incidence of polyspermy in spite of the fact that more than 2 dozen sperm are attached, visually. Under normal conditions, one would not expect a high degree of polyspermy at these sperm/egg ratios (5). However, in our experiments the sperm are already attached and blocked at the electrically sensitive step and theoretically all the attached sperm are in the same state of readiness. One always sees a successful activation if the electrical block is released even several minutes after sperm addition. The simplest explanation is that sperm can oscillate in the degree of readiness to respond to the period of inside negativity and that at the durations and sperm concentrations used in these experiments only one sperm was ready.

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