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Bioelectric Responses of the Echinoderm Egg to Fertilization

(fertilization/sea-urchin egg/membrane potential/sodium permeability/intracellular potassium/potassium permeability)

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ABSTRACT **The fertilization reaction of echinoderm eggs (*Lytechinus pictus*, a sea urchin, and *Dendraster excentricus*, a sand dollar) was followed with intracellular electrodes. Membrane potential and K^+ activity were recorded.**

The unfertilized egg of *Lytechinus* has a membrane potential of −8 mV, inside negative. Within 5 sec after the addition of sperm, a *fertilization action potential* develops, going to +10 mV, inside positive. The time from the initial depolarization to a return to the original −8 mV is 120–150 sec. The repolarization continues until a potential of −10 to −14 mV is reached, at which point it pauses for 3–4 min. At 6–8 min after fertilization, a further and relatively rapid hyperpolarization begins, going to −60 to −65 mV by 15–25 min after fertilization and remaining constant at these values.

The membrane potential of the unfertilized egg appears to depend on a general permeability to anions. The fertilization action potential seems to reflect a prolonged influx of sodium. The final depolarization to −60 mV is attributable to the development of potassium conductance.

Simultaneous measurements with a K^+ ion-selective electrode gives constant readings of about 240 mM K^+ in the unfertilized eggs throughout the fertilization process.

Similar results were obtained with *Dendraster* eggs. The resting potential of the unfertilized eggs was −7 mV; the action potential on activation attained +18 mV; the repolarization paused at −16 to −24 mV and the final potential attained was −70 mV. The electrical changes after fertilization with spermatozoa or activation with Pronase were identical.

This report deals with alterations in membrane potentials accompanying the fertilization of echinoderm eggs. The problem has a long history, associated with the intuition that the activation of the egg should have some analogies with the responses of recognized types of excitable cells to stimulation. An electrical response at fertilization was predicted by Lillie (1); the difficulty of recording it was discussed by Rothschild (2) and, in more recent times, there has appeared some evidence that the membrane potentials of animal eggs do change during the activation of the eggs (3). We now report and interpret a definite sequence of bioelectric changes during the activation of sea urchin and sand dollar eggs, including a response analogous to an action potential.

Previous studies had been hampered by the difficulty of penetrating the extremely flexible plasma membranes with microelectrodes. The solution of various technical problems permitted us to make more than 150 successful recordings under conditions where the ionic medium surrounding the eggs could be varied; thus, the permeability characteristics of the membranes at various stages of the fertilization process could be analyzed. Our interpretations of the membrane changes have also been reinforced by simultaneous measurement of intracellular K^+ ion activity and membrane potential.

MATERIALS AND METHODS

Handling of gametes

Eggs and sperm of the sea urchin *Lytechinus pictus* and the sand dollar *Dendraster excentricus* were obtained by the injection of 0.5 M KCl into the coelomic cavity or by dissection. The jelly coats were removed from the eggs by passing them through fine-meshed silk. The penetration of the eggs by electrodes was less reliable after removal of the jelly coats with acidified sea water.

Fertilization and parthenogenetic activation

In experiments in normal sea water, fertilization was achieved simply by dropping a concentrated suspension of sperm into the dishes in which an egg was impaled on electrodes. Fertilization by spermatozoa was often difficult, and sometimes impossible, in media other than normal sea water because of the inactivation of the spermatozoa. *Lytechinus* spermatozoa were somewhat more tolerant and successful fertilization was achieved in low-Na sea water (glycerol or glucosamine·HCl substituted for NaCl); low-Cl or Cl-free sea water (isethionate, sulfate, or nitrate substituted for Cl) and low-K sea water. The formulations are given below. *Dendraster* spermatozoa were somewhat more sensitive to the abnormal media, but were very suitable for experiments with parthenogenetic activation. For this purpose, we introduced the proteolytic enzyme Pronase (Calbiochem) at a concentration of 20–30 μg/ml in whatever medium was being tested. *Lytechinus* eggs could not be activated by Pronase (4). Some information on the bioelectric events in the activation of the *Lytechinus* egg was obtained by the use of the classical Loeb method—about a 90-sec exposure to 5×10^{-3} N butyric acid followed by the replacement of the butyric acid with normal sea water. However, the electrode usually came out of the egg in the course of this treatment and had to be reinserted, with a loss of part of the record.

The ease of activating the *Dendraster* egg not only made it possible to compare bioelectric changes in fertilization and artificial activation, but also permitted the study of activation in media in which fertilization by spermatozoa is impossible.

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Immobilization of eggs for micromanipulation

The difficulties of inserting electrodes into echinoderm eggs have been well described and illustrated (2, 3). The surface of the egg yields to the electrode tip, and an additional mechanical impetus is needed to puncture the membrane and to seat the tip inside the cytoplasm. The egg must be held very firmly, else it will roll or tear. Successful penetrations have been achieved in the past by holding a single egg on the "micro-sucker", or by supporting them in an agar gel (3).

We have found a simple solution of the problem by taking advantage of the negative charge on the surface of the eggs (5); in effect, the egg is treated as a giant anion.

The containers used for our operations were 60 × 15 mm plastic Petri dishes (Falcon Plastics, Los Angeles). The bottoms of these dishes were conditioned merely by covering them with a 1% solution of protamine sulfate. The protamine solution was washed away leaving a very hydrophilic surface, presumably coated with a positively charged monolayer of protamine. The eggs, in whatever medium was being used in a given experiment, were dropped into such coated dishes containing the experimental solutions. When they settled, they attached strongly, flattening themselves against the surface. With these preparations, the electrodes remain seated in the eggs despite the turbulence produced when sperm suspensions or experimental solutions were added.

For experiments in which the solutions had to be changed while potentials were being recorded, a simple "chimney" device that provided laminar flow of solution across the bottom of the dish was improvised. A cylinder about 30 mm high was cut from a 15 mm (outside diameter) polyethylene centrifuge tube. A flat notch, 1–2 mm high and about 10 mm wide was cut at the end of the cylinder. This "chimney" was affixed to the bottom of the petri dish with vaseline, with the notch facing the center of the dish. When solutions were added through the top of the "chimney" they came out through the notch and flowed across the bottom of the dish, displacing the medium around the impaled egg.

Solutions

Artificial sea water was composed as follows: NaCl, 484 mM; KCl, 10 mM; $MgCl_2$, 27 mM; $MgSO_4$, 29 mM; $CaCl_2$, 11 mM; $NaHCO_3$, 2.4 mM. The pH was about 8.2. "Unbuffered sea water" was the above mixture with $NaHCO_3$ omitted. Its pH was around 6, depending on exposure to atmospheric CO_2.

Low-sodium sea waters were made by the replacement of NaCl in the above formula with osmotically equivalent amounts of erythritol, glycerol, or glucosamine (free base, neutralized with HCl). Many other replacements such as glucose, sorbitol, mannose, glycine, etc. were found to be unsuitable because they were extremely harmful to eggs or spermatozoa, especially to spermatozoa. Some batches of erythritol were harmful for unknown reasons. Although glycerol is known to penetrate echinoderm eggs, the permeability was low in the species we used and little or no swelling was observed during experiments lasting 30 min or less.

Low Cl sea water was made by the use of Na isethionate in the place of NaCl. *Cl-free sea water* was made by substituting sulfates for chlorides in the artificial sea water. The formula was: Na_2SO_4, 320 mM; K_2SO_4, 6.6 mM; $MgSO_4$, 55 mM; $NaHCO_3$, 2.4 mM. Ca was omitted because of the insolubility of $CaSO_4$.

Nitrate sea water was made by substituting $NaNO_3$ or KNO_3 for the Cl^- ion salt of Na and K.

Butyric sea water contained 5×10^{-3} N butyric acid in artificial sea water.

Low K and high K sea waters were made by appropriate substitutions of NaCl for KCl and vice versa.

Recording of potentials

Membrane potentials were recorded in the conventional manner with glass microelectrodes filled with 3 M KCl in series with a high impedence unity-gain preamplifier and a Tektronix 564 memory oscilloscope. Microelectrode tips that gave resistances of less than 15 MΩ were generally too large for successful penetration of the cell membrane. (The diffusion potential from the microelectrode tips was always less than 5 mV.) The indifferent electrode was a KCl–Calomel electrode with a 3 M KCl agar bridge. Voltages recorded were calibrated by a Bioelectric Instruments Calibrator Model CA5.

K^+ ion activity*

K^+ ion-selective microelectrodes were made by filling regular glass microcapillaries with Corning ion-exchanger 477317, after treating the tips with a siliconing agent (6). These K^+ ion-selective microelectrodes usually gave a 54 mV response to a 10-fold change in K^+ ion that was linear over the range of 10–400 mM K^+ ion, while only giving a 2 mV signal to a 10-fold change of Na^+ ion. The K^+ ion-selective microelectrodes were insensitive to pH over the range 5–9 and gave no measurable response to changes in Ca^{++} from 0.1 to 10 mM. In making estimates of intracellular K^+ ion activity, the membrane potential recorded simultaneously by another microelectrode was subtracted from the voltage of the K^+ ion-selective microelectrode to yield the voltage due to K^+ ion activity alone. K^+ ion-selective microelectrodes were calibrated in solutions roughly corresponding to the intracellular medium (7) before and after each series of measurements. Because of the high impedence of these ion-selective electrodes, potentials were measured by an Analog Devices (Cambridge, Mass.) model 311K operational amplifier, which has more than 2×10^{14} Ω input impedence in a unity gain configuration.

RESULTS

Membrane potential of the unfertilized egg

Upon penetration of an unfertilized egg, we observe a sudden potential drop of 5–10 mV. Frequently some voltage is lost momentarily, but the potential is restored and remains stable at −7 to −8 mV for a long time.

The membrane potential of the unfertilized egg is insensitive to cations and sensitive to anions, as is shown in Table 1. Substitution of other anions for Cl lowered the potential. In the case of the substitution of SO_4 for all the Cl in the sea water, the potential was reversed to +5 mV.

Larger resting potentials were obtained when the unfertilized eggs were placed in sea water containing butyric acid (5×10^{-3} N) or exposed to CO_2.

All these observations lead to the conclusion that the membrane of the unfertilized egg is anion permeable, and that the resting potential depends on the Cl gradient. In a series of

* Electrodes of the type used in the present work are now available from Frederick Haer and Co., P.O. Box 2138, Ann Arbor, Mich. 48106.

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TABLE 1. *Resting potential of the unfertilized egg*

mV*	Medium	Species	No. of cells
	CATIONS		
−8	sea water	L	20
−7	sea water	D	11
−8	10–25% sea water/ 90–75% glycerol sea water	D	12
−8	0 mM K^+ ion	L	7
−7	200 mM K^+ ion	L	12
−7	0–2 mM K^+ ion	D	10
−8	60–80 mM K^+ ion	D	3
−6	200 mM K^+ ion	D	10
	ANIONS		
−3	Na acetate	L	14
+1	Na isethionate, K isethionate, $MgSO_4$	D	6
+5	All SO_4, no Ca^{++} ion	D	11
−25	CO_2 or butyric acid in sea water	L	8

* These are average values. All data obtained were within a 3-mV range of the averages. L, *Lytechinus pictus;* D, *Dendraster excentricus.*

experiments on *Dendraster*, it was observed that the substitution of NO_3 for Cl in the sea water increased the resting potential to as high as −16 mV, suggesting that the membrane is more permeable to NO_3 than to Cl. The increase in the potential to −25 mV in the presence of butyric acid or CO_2 is another indicator of an anion-permeable membrane (6, 8). However, the normal magnitude of about −8 mV is small for the about 10-fold Cl gradient between the sea water and the interior of the egg, assuming that the published values for the chloride content of the egg (7) are correct. We picture a membrane impermeable to cations, and having a low permeability to Cl, which is increased in the presence of weak organic acids.

Membrane potential changes upon activation; the three phases

Immediately upon activation, there occurs a depolarization that we term the *fertilization action potential.* The membrane potential is reversed to +10 mV in the *Lytechinus* egg and to +18 mV in the *Dendraster* egg. The repolarization takes the potential to a level below the resting potential of the unfertilized egg, at which point it pauses. We will refer to the period of the fertilization action potential as Phase I. Phase II is the pause during which the negative potential is increasing only gradually. After the pause, a rather rapid hyperpolarization to its final level sets in; we term this Phase III. At the end of Phase III, the potential is about 60 mV in the *Lytechinus* egg and about 70 mV in the *Dendraster* egg and remains at these levels for some time; our measurements only extend to the 8-cell stage. The times of initiation of these phases after activation are rather constant, although the exact time course of the potential changes within each phase is slightly variable from cell to cell.

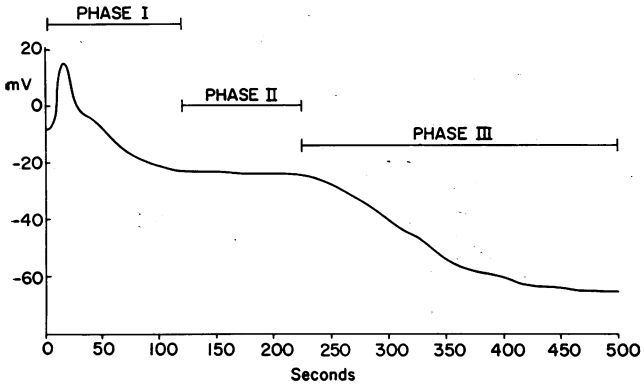

FIG. 1. Membrane potentials after activation of *Dendraster* egg with Pronase. Identical results are obtained when fertilizing with sperm. See text for explanation of the three phases.

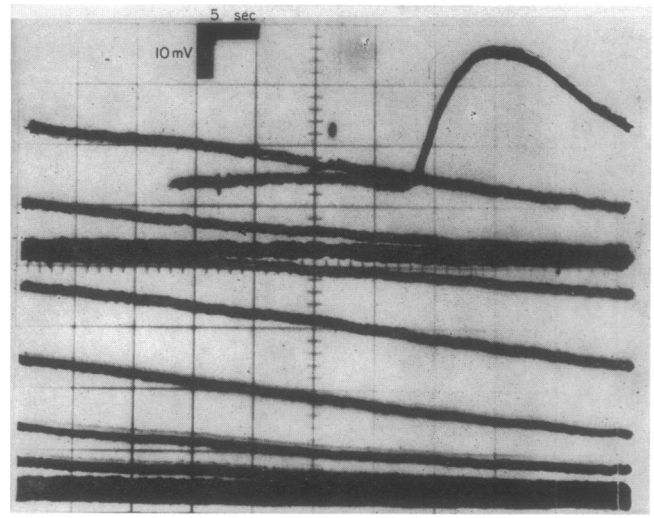

FIG. 2. Membrane potentials after activation of *Dendraster* egg with Pronase. Record of superimposed traces from a storage oscilloscope showing experiment displayed graphically in Fig. 1. Resting potential of the unfertilized egg is indicated by level line below *dot.* The *dot* indicates zero membrane potential. Downward deflection indicates negative potentials measured intracellularly. Reading from right to left, we see in turn the resting potential of the unfertilized egg, the depolarization and inside positive potential of the fertilization action potential or Phase I, the recovery and pause of Phase II, and finally the sudden development of the high potentials of Phase III.

Representative data on the course of these events is displayed in Fig. 1 (activation of *Dendraster* egg with Pronase).

Fertilization action potential: Phase I

If care is taken to insure rapid mixing within 3 sec after the addition of sperm or activating agent, the membrane potentials of either *Lytechinus* or *Dendraster* eggs rapidly depolarize. The depolarization continues beyond zero membrane potential, developing substantial inside positive voltages. In *Lytechinus* eggs, the absolute amplitude of the *fertilization action potential* commonly amounts to 18 mV, a change from −8 mV to +10 mV. In *Dendraster* eggs the absolute amplitude commonly reaches 25 mV, going from −7 mV to +18 mV.

Fig. 2 shows the superimposed memory oscilloscope traces from an experiment in which *Dendraster* eggs were activated with Pronase. The expanded time scale of Fig. 2 shows the details of the depolarization and the other membrane potential changes that follow during a typical fertilization or activation reaction. The phases generally proceed more rapidly in *Dendraster* than in *Lytechinus* (Fig. 3), but in both species the fertilization action potential is quite slow compared to

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action potentials of nerve and muscle cells. The initial depolarization takes over 10 sec to complete and the recovery, a gradual repolarization, takes more than 100 sec.

Low Sodium Solutions. The amplitude of the depolarization of phase I is greatly reduced by substituting large solute mole cules for Na^+ ions in artificial sea-water solutions. Fig. 4 shows the reduced *fertilization action potential* attained when activating *Dendraster* in a solution comprised of 25% normal sea water/75% glycerol sea water. The same results are obtained when *Lytechinus* eggs are fertilized in 25% normal sea water/75% glucosamine·HCl; the amplitude of Phase I depolarization is reduced but the subsequent phases appear to proceed normally.

The Phase I changes are completely abolished when the Na gradient is eliminated by reducing the Na level to 10% of normal. This observation applies to the activation of *Dendraster* eggs with Pronase or the fertilization of *Lytechinus* eggs with spermatozoa. At this level of Na, Phase II develops more slowly than is normal and Phase III often terminates at 30–50 mV instead of the normal 60–70 mV.

The pause: Phase II

The pause that follows Phase I typically occurs at minus 10–14 mV and lasts until 6–8 min after fertilization in *Lytechinus*. In the activation of *Dendraster*, the pause reaches minus 16–24 mV and only lasts 4–5 min after fertilization. This is the phase of the fertilization reaction that is the most variable in magnitude of potential and in duration. A slow Phase I and a quick onset of Phase III can obliterate any obvious sign of Phase II. Phase II is always more clearly separated in the fertilization reactions of *Dendraster* due to the higher voltage attained, but here again there is a large variability in these voltages. Toward the end of the season (September 1970) *Dendraster* Phase II often reached minus 30–40 mV.

The development of K^+ ion conductance: Phase III

The end of Phase II is clearly marked by a sudden acceleration in the hyperpolarization at 4–5 min in *Dendraster* and 6–8 min in *Lytechinus* fertilization reactions. The hyperpolarization reaches minus 60–65 mV in 15–25 min in *Lytechinus* and minus 70–80 mV in 8–12 min in *Dendraster* (Figs. 1–4).

Changes in External Potassium. High potassium sea waters (60 mM K^+ ion substituted for equivalent Na^+ ion) reduced Phase III voltages to minus 30–40 mV in both *Lytechinus* and *Dendraster*. Additional substitution of K^+ ion for Na^+ ion reduced the Phase III voltages further, approaching zero membrane potentials as the concentration of potassium reached 200 mM. In low-potassium sea waters (<2 mM K^+ ion), Phase III commonly attains minus 90–110 mV.

Intracellular K^+ Ion Activities. In three cases each of *Lytechinus* and *Dendraster* activations entire fertilization reactions were monitored with intracellular K^+ ion-selective microelectrodes. Penetration of the plasma membrane with K^+ ion-selective microelectrodes results in a positive deflection to plus 55–65 mV, which corresponds to an intracellular K^+ ion activity in the range 200–240 mM when the corrections for membrane voltage are made and the resulting figures are compared to the voltages from calibration solutions. By simultaneously monitoring membrane voltages with ordinary microelectrodes and K^+ ion activity plus membrane voltage with K^+ ion-selective microelectrodes, we could follow any

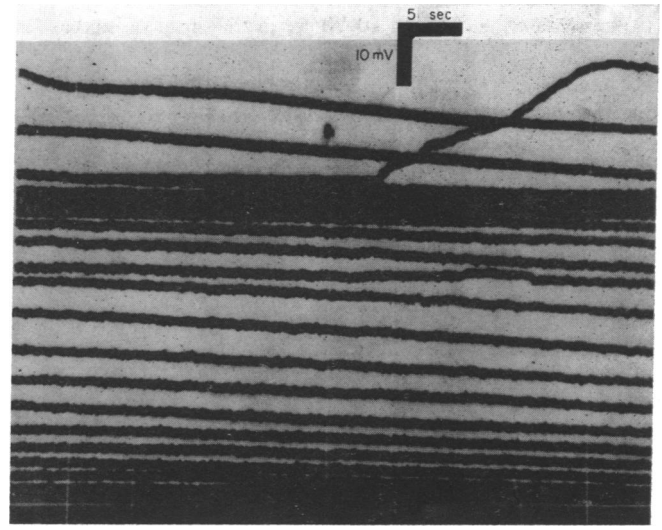

FIG. 3. Membrane potentials after fertilization of *Lytechinus*. Superimposed traces from a storage oscilloscope. See legend of Fig. 2.

changes in K^+ ion activities during the fertilization reaction. The only slight changes observed corresponded to the few millivolts lag of the K^+ ion microelectrode relative to the potential-recording electrode due to the very high ($>10^{12}$ Ω) impedence of the former. This result rules out K^+ ion accumulation or decompartmentalization and suggests that phase III indicates a change in membrane conductance.

DISCUSSION

The interpretation of metabolic inhibition of the unfertilized egg can now be extended to include control of ionic permeabilities of plasma membranes. There may be an energetic advantage in turning off the potassium conductance in a maturing egg that may be held for months before release and fertilization. The action potential does seem to be the immediate response to activation; it has been seen within 3 sec. There may be no lag apart from the time required for spermatozoa or solutions to reach the surface. All the other events described in the large literature come later. Recently, Nakazawa *et al.*

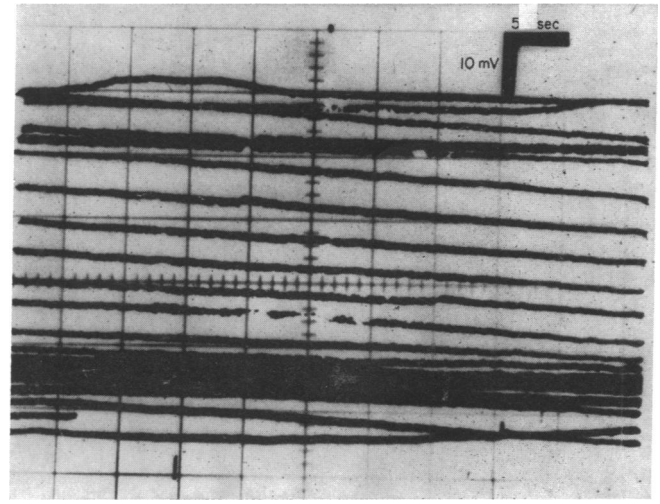

FIG. 4. Membrane potentials after activation of a *Dendraster* egg in 25% sea water/75% glycerol sea water. Note the nearly complete obliteration of phase I. See legend of Fig. 2 and text.

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(9) have recorded a lag of 10–20 sec in the onset of increase in respiration at fertilization. Paul and Epel (10) report that the changes in light scattering indicative of the "cortical reaction" take place 40–60 sec after fertilization.

However, it does not follow that the influx of Na, which is the event that our experiments with ion-substitution detects, is important in itself for the triggering of subsequent events. It may only reflect changes in the membrane that affect the activities of other ions or molecules.

We have no basis at present for assigning a particular ionic mechanism for the enhanced membrane potentials observed during Phase II, which were more difficult to work with sandwiched, as they were, between the other two fairly rapid transitions. The timing of Phase II does, however, suggest that it reflects the fusion of cortical granule membrane with the egg membrane. Earlier work has shown a large increase in membrane capacitance after fertilization that supports this view (11). Other possibilities remain, however, such as an electrogenic efflux of sodium triggered by the preceding influx.

We have made a few preliminary measurements of membrane resistance that support our interpretation of the potential changes after activation. Phase I is always accompanied by a large drop in resistance. Phase II involves a recovery and Phase III always returns the resistance to very low values. However, we do not yet have complete confidence in absolute values for resistance since they depend to a much larger extent on good membrane healing after penetration with two microelectrodes.

Phase III is clearly a period in which the membrane is developing its K^+ ion conductance. At the end of Phase III, the egg has a typical K^+ ion-selective membrane, responding to external K^+ concentration according to theory. The time of onset of Phase III corresponds to the time at which increased amino-acid uptake and increased protein synthesis begins (12), but we have no evidence of a causal relation between the two. However, our measurements of intracellular K^+ activity, which remains constant, do not support the proposal (13) that decompartmentalization of K^+ is linked to the initiation of protein synthesis.

In summary, the fertilization reaction includes a series of changes in the cell membrane that begins instantly with an action potential and requires a relatively long time to go to completion. The membrane of an unfertilized egg that is impermeable to cations and has a low permeability to anions is converted into a typical membrane whose resting potential is accounted for by its permeability to potassium.

NOTE ADDED IN PROOF

A depolarization of the egg membrane at the time of fertilization has been described recently by Morrill, G. A., A. B. Kostellow, and J. B. Murphy, *Exp. Cell Res.* **66,** 289 (1971) for the frog egg and by L. Jaffe (personal communication; *Develop. Biol.*, in press) for the egg of the alga *Fucus*.

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