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RETURN TO NORMAL OF *FUCUS* EGG MEMBRANE AFTER MICROELECTRODE IMPALEMENT

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SUMMARY

Continued microelectrode impalement of developing *Fucus* eggs for ½ h is accompanied by three major changes:

1. A ten-fold increase in membrane resistance (together with a marked *decrease* in the membrane potential's response to potassium concentration changes).

2. The appearance of spontaneous episodes of depolarization about 100 sec in length and 2–6 mV in amplitude.

3. A disappearance of the 'hyperpolarizing response', i.e. of the rapid increase in resistance produced by inward current. The resting potential, on the other hand, scarcely changes during impalement.

These results are compared with those from non-destructive studies of this membrane's behavior (those of tracer ion fluxes and with extracellular recording), as well as with the literature on other cell types. These comparisons indicate that this membrane has been somehow disturbed by the act of impalement and only slowly returns to a relatively normal state during continued impalement. The symptoms of this disturbance indicate that it involves a large and selective increase in potassium permeability. We speculate that this increase in P_K is in turn produced by an increase in the concentration of cytoplasmic calcium during the impalement process.

Our interest in accurate and relevant information on the membrane properties of fucoid eggs is founded in the general hypothesis that development is a centripetal process, in which the cell surface changes first and the cell nucleus last [6]; in the more specific hypothesis that membrane-generated cation currents and self-electrophoresis are major mechanisms of intracellular localization processes [8]; and in the specific evidence for the operation of this process in polarizing fucoid eggs [7].

Our first study of these eggs using intracellular microelectrodes yielded some puzzling discrepancies with other data [12]. In particular, a tracer flux study indicated membrane resistances 10 to 50 times higher than those indicated by intracellular microelectrodes [10]. In 1970, Spanswick reported that "the membrane resistance of internodal cells of *Nitella translucens* increased by 50 % during the first 5 h after insertion of two microelectrodes"; moreover, the final and higher resistance value was closer to that estimated from ion flux measurements than the initial value [11]. This observation helped suggest to us that some slowly dissipated impalement disturbance might lie behind the discrepancies observed in fucoid eggs. The present study confirms this suspicion and delineates a relatively subtle puncture artifact that may be of more general significance to all research which involves puncturing cells with micropipettes.

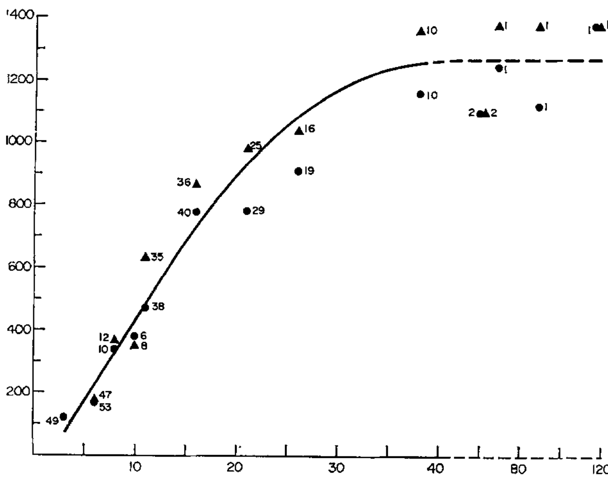

Fig. 1. Abscissa: time after impalement (min); *ordinate:* specific membrane resistance (ohm·cm²).
Specific membrane resistance during continued impalement of one-day-old, germinated eggs of *Fucus serratus.* The eggs were punctured with the first micropipette at time 0; with the second one at 1–3 min. All measurements were made with single depolarizing (●) or hyperpolarizing (▲) current pulses of 10–50 msec duration which displaced E by less than 20 mV. Numbers next to symbols show the number of cases averaged. Time scale is compressed five-fold after 40 min.

MATERIALS AND METHODS

For the present investigation we used gametes of the dioecious seaweed *Fucus serratus.* The thalli of this brown alga were collected at Helgoland in the North Sea and stored semidry at 2–5°C for up to 3 weeks. The joint release of eggs and sperms was induced by osmotic shock [9]. Zero time for development was taken to be 1 h after shedding began. The zygotes were grown and studied at 15±2°C. Except during measurements they were under continuous white fluorescent light. The germination rate varied between 4 and 95 %. The average diameter of an egg is 78 μm.

The techniques for immobilizing the eggs, fabrication of glass microelectrodes and impaling an egg with two micropipettes were essentially the same as described earlier [12]. In sea water, the micropipettes filled with 3 M KCl had tip resistances of 10–20 MΩ (those filled with 0.3 M KCl, 50–70 MΩ). All pipettes used had tip potentials of less than −10 mV in sea water. The recording equipment consisted of a preamplifier (Bioelectric Instr.), a storage oscilloscope (Tektronix) and a chart-recorder (Sargent). A Grass stimulator in series with a 10^9 Ω resistor served as a current source. Current was injected into an egg through a second micropipette pushed into the cell 1–3 min after the first one and oriented about 50–60° to the former. Several single d.c. pulses of increasing strength and 10–50 msec duration were applied at intervals commencing ½ min after the penetration. The sequence of the current directions was normally outward–inward–outward ... Measurements of the membrane resistance were carried out as long as the membrane potentials were fairly stable. After 20 min, an increasing fraction of the eggs' membranes became grossly and increasingly depolarized and conductive. We believe the latter had been torn and damaged by some unavoidable movements of the electrodes and the egg support system. In any case, we felt justified in considering them grossly damaged, and in restricting further measurements to the residual, relatively stable cells. As can be seen in fig. 1, while the number of cells showing such stable behavior declined rapidly after 30 min, enough remained for as long as 1–2 h to provide evidence of a reproducible pattern of recovery from impalement.

The artificial sea water used throughout the experiments had the same composition as in previous studies [12]. In media with high K^+, it was increased at the expense of Na^+. In media with low K^+, it was replaced by Na^+.

RESULTS

Resting potential of developing eggs

The average membrane potential, E, of 134 germinated, one-day-old *F. serratus* eggs, 1 min after penetration of the first (voltage measuring) pipette was −78 mV. This value is equal to the figure of −78 mV previously reported for one-day-old *F. serratus* eggs [2] and close to that of −72 mV for the closely related *Pelvetia fastigiata* eggs [12]. Upon penetration of the second (current carrying) pipette, the membrane depolarized between 10 and 40 mV, and then, during the next 1–3 min, recovered to a value depolarized by an average of only about 2 mV below the first stable value. Later changes were very small. This observation suggests that a similar episode of gross and transient membrane depolarization occurs at the first impalement,

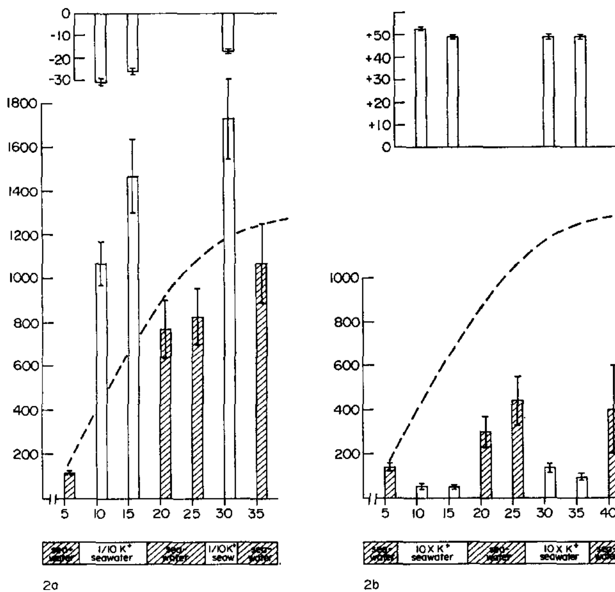

Fig. 2. Abscissa: time after impalement (min); *ordinate:* (*top*) change in membrane potential (mV) (+ indicates depolarization; −, hyperpolarization); (*bottom*) specific membrane resistance (ohm·cm²).
Changes in membrane potential (*top*) and of membrane resistance (*bottom*) of one-day-old *F. serratus* eggs with changes in the artificial sea water's K^+ concentration during continued impalement. Duration and time of application of the various media is indicated below the graphs. ---, Typical course of the membrane resistance in sea water.

too. In the period from 20 to 120 min after impalement, the average value of E did not differ by more than 2 mV from the value measured 5 min after puncturing. And so did those made with micropipettes filled with 0.3 M KCl instead of the usual 3 M KCl.

Resistance of developing eggs

Measurements of the specific membrane resistance, R, of one-day-old, germinated *F. serratus* eggs a few minutes after impalement gave an average value of about 100 ohm · cm², a result similar to that previously reported for *P. fastigiata* [12]. But when we tried to monitor the membrane resistance during continued impalement, we were surprised to find it rising greatly. Fig. 1 shows the results of an extended study of this phenomenon. During the first half hour of impalement, R rose at 30–40 ohm · cm² · min^{-1}, and after 40 min of impalement, it had risen about ten-fold to reach a steady value of about 1 200–1 400 ohm · cm². A few measurements on zygotes at 6–10 h after fertilization revealed similar results. So did measurements on one-day-old eggs kept in sea water at pH 6 instead of the normal pH 8 for ½ h.

Responses of developing eggs to changes in K_0^+

Fig. 2 shows the changes in E and R as impaled eggs are alternately immersed in media containing the natural value of 10 mM K^+ and those with 1 mM K^+ (fig. 2*a*), or 100 mM K^+ (fig. 2*b*). The potassium sensitivity of E falls during impalement. In the period from 10 to 30 min after first puncture, the hyperpolarization produced by 1/10 K^+ sea water falls from 30 ± 1 mV to 17 ± 1 mV; while the depolarization produced by $10 \times K^+$ sea water falls from 53 ± 1 mV to 49 ± 1 mV. R is roughly doubled by 1 mM K^+ sea water. Upon a return to a 10 mM K^+ sea water it quickly returns to the value found in eggs kept in a 10 mM K^+ medium for the same length of time after impalement. On the other hand, R is reduced about ten-fold by 100 mM K^+ sea water; but upon a return to a 10 mM K^+ sea water it does not soon

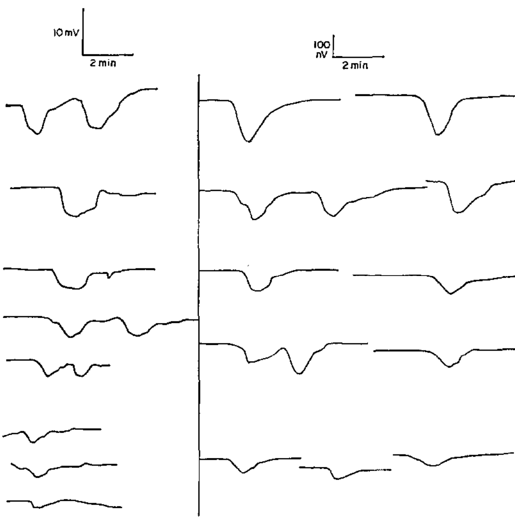

Fig. 3. (*Left*) Spontaneous episodes of membrane depolarization in one-day-old *F. serratus* eggs recorded with an intracellular micropipette. Downward deflections indicate depolarization. The resting potentials varied from −70 to −75 mV. (*Right*) Representative spontaneous current pulses through one-day-old *P. fastigiata* eggs recorded with an extracellular vibrating electrode. To facilitate the comparison of durations, both types of recordings have been drawn to the same time scale. To facilitate the comparison of shapes, the vertical scales of the two kinds of recordings has been set to give about the same heights. The vertical scale on the right indicates the difference in nV measured by an electrode vibrating with a 30 μm amplitude, between two points in the sea water 35 and 65 μm in front of the egg's rhizoid pole. The measured voltage difference is divided by 30 μm times 24 ohm·cm^2 (the sea water's resistivity) to convert it into a measured current density value, $\delta_M \cdot \delta$ at the rhizoid's tip is estimated to be eight times greater than δ_M by an extrapolation procedure. Thus a 100 nV pulse amplitude indicates about 10 μA/cm^2 entering the rhizoid's tip. Data taken from another investigation [7, 8].

recover the high value found in eggs kept in a 10 mM K$^+$ medium.

Spontaneous episodes of depolarization in developing eggs

In 11 cases we recorded the entire course of a developing egg's membrane potential during impalement. An examination of these records revealed 14 clear episodes of transient depolarization, most of which are shown in fig. 3. These episodes lasted 1 to 2 min, had peak amplitudes of 2 to 6 mV, and depolarized faster than they recovered. Their times of occurrence after first puncture are shown in fig. 4.

There is a clear and significant absence of such episodes until 20 min after first puncture. Between 20 and 60 min after puncture, 12 episodes were recorded in a total time of 5.2 h, thus 2.3 episodes/h; while between 5 and 20 min after puncture, zero episodes were recorded in 2.8 h. Yet at a rate of 2.3 episodes/h, a total of 6.3 episodes would have been expected to occur on the average in these 2.8 h. The theoretical chance of the observed complete absence of events being the result of an accidental fluctuation should be given by $e^{-6.3}$, or about 0.001.

Responses of developing eggs to inward current

We have previously reported that developing *Pelvetia* eggs can show strikingly hyperpolarizing responses; i.e. they may increase their membrane resistance about five-fold some msec after an inward current hyperpolarizes them by 20 mV or more [12]. We soon found this same phenomenon in *F. serratus* eggs and illustrate it in the left

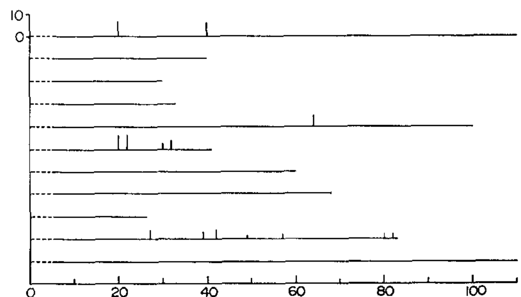

Fig. 4. Abscissa: time after first impalement (min); *ordinate:* peak depolarization during an episode (mV).

Spontaneous depolarization episodes in 11 separate one-day-old *F. serratus* eggs. (Impalement artifacts would have precluded observation of episodes before 5 min.)

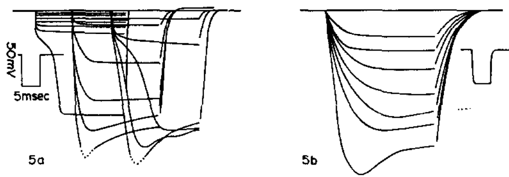

Fig. 5. Representative membrane potential responses of germinated *Fucus* eggs to inward current pulses. (*a*) (Left traces) cell in sea water, 5–6 min after puncturing; current (from top): 32 – 72 – 156 – 200 – 247 – 296 – 343 μA/ cm²; (middle traces) same cell in 1/10 K⁺-sea water which hyperpolarized the membrane by 21 mV; current: 32 – 72 – 112 – 156 – 200 μA/cm²; (right traces) same cell back in sea water; current: 32 – 72 – 80 – 92 – 112 μA/cm²; (*b*) response of a cell in sea water when *R* has increased to about 2 000 ohm·cm²; current: 21 – 30 – 40 – 49 – 58 – 66 – 112 μA/cm². A tracing of the response to a calibrating square pulse is given next to each figure.

trace of fig. 5*a*. Then we found a significant new fact: The egg's membrane only yields a hyperpolarizing response in a state of relatively low resistance; either soon after impalement in a medium with the natural 10 mM K^+ concentration, or at any time after impalement in 100 mM K^+ sea water. It will not yield a hyperpolarizing response in a state of high resistance. Either after prolonged impalement in a 10 mM K^+ sea water (fig. 5*b*) or at any time in a 1 mM K^+ sea water (fig. 5*a*, middle trace), it behaves like a relatively fixed resistor. (More precisely it behaves this way up to a membrane potential of about −250 mV. Above this, 'punch through' seems to occur.) The transition between the two states occurs at about 1 000 ohm · cm².

Observations on unfertilized eggs

The average membrane potential of ten unfertilized eggs was −33 mV during the first minute after first puncture (maximum −43 mV). Upon penetration of the second (current-carrying) pipette, the membrane depolarized by about 20 mV, then recovered only very slowly. After about 30 min of double impalement, it had recovered, i.e. repolarized, by an average of only 8 mV. The average specific membrane resistance of these eggs was 1 200 ohm · cm² soon after second puncture, then slowly declined to 900 ohm · cm² during 30 min of continued impalement. Unlike the membrane of developing eggs, that of the unfertilized egg seemed entirely passive; it showed no rectifying properties, no delayed responses to current flow, and no spontaneous episodes of depolarization.

DISCUSSION

Evidence that the membrane of the developing egg approaches a normal state during impalement

We observed several major changes in the electrical behavior of developing *F. serratus* eggs during a half hour of impalement. Our analysis indicates that the egg's membrane is returning to a normal state during impalement, rather than degenerating. Three main considerations support this contention.

First, the specific membrane resistance of the day-old eggs of the closely related species, *Pelvetia fastigiata*, has been estimated to be about 3 000 ohm · cm² from tracer flux measurements [10]. This value is far closer to the 1 400 ohm · cm² approached by *F. serratus* eggs during prolonged impalement than the 100 to 200 ohm · cm² seen soon after puncture. The tracer flux method is obviously non-destructive and thus experimentally reliable. It is also true that there are a number of theoretical uncertainties in converting a tracer flux value to a resistance value, but to our knowledge the only one which could over-estimate the resistance is the single-file effect; and there is no evidence that such effects can provide more than two-fold errors [4]. So comparison with the resistance values yielded by the non-destructive tracer flux method is one strong evidence that the

developing egg's membrane approaches a normal state during prolonged impalement.

Second, non-destructive extracellular recording (with a vibrating electrode) shows that one-day-old *P. fastigiata* eggs drive spontaneous pulses of current through themselves. The current in each transcellular pulse moves in a loop which enters an egg at its growing point, crosses its cytoplasm, leaves at some non-growing point and returns through the medium [7, 8]. As fig. 3 (right) shows, these externally recorded pulses have a similar duration and shape to the internally recorded depolarization episodes. They occur at a similar frequency of a few per hour. Moreover, a third of the externally recorded pulses occur in 'twins', i.e. pairs of nearly equal size which follow each other within 10 min or less, while 6 to 8 of the 14 recorded episodes likewise occur in such twins. Furthermore, a crude calculation indicates that the peak amplitudes of the depolarization episodes are of about the size that should accompany the observed transcellular current pulses [8]: The latter indicated peak intracellular current densities, δ of the order of 1 to 10 $\mu A/cm^2$. The membrane depolarization produced by the flow of such a current out of the (presumably passive) thallus pole of an egg is given by $\delta \times R$, thus about 10^{-6} to 10^{-5} $A/cm^2 \times 10^3$ $ohm \cdot cm^2$ which is 1 to 10 mV.

Altogether, then, there is little doubt that the episodes of depolarization recorded with an electrode inside *F. serratus* eggs indicate the same kind of transcellular current pulses recorded outside of *P. fastigiata* eggs by a non-destructive method. The depolarization episodes were absent until 20 min of impalement had passed, then appeared at the frequency of transcellular current pulses. So this is further substantial evidence that the membrane's behavior is first seriously disturbed by impalement and then returns to a relatively normal state.

Third, the disappearance of the hyperpolarizing response during impalement in normal sea water also suggests a return to a relatively normal state. For with the exception of the highly aberrant membrane of the *Hypopomus* electrocyte, we know of no other cell membrane which shows a rapid hyperpolarizing response except in media with elevated potassium concentrations. (This point is documented in table 6 of reference [12].)

Nature of the disturbance produced by micropipette insertion

We find about a 40 mV/decade response of the recently impaled, one day old *F. serratus* egg to a change in K_0^+. (About the same response has been reported for 12–14 h old *F. serratus* eggs [2] and for one-day-old *Pelvetia* eggs [12].) It is therefore clear that most of the conductance measured soon after impalement is due to potassium ions. Moreover, the potassium sensitivity of the freshly impaled egg's membrane is actually greater than that of the long-impaled one. Hence the ten-fold increase in conductance produced by puncture must be mediated by a comparable and relatively selective increase in potassium conductance.

How can puncture produce a selective and only slowly reversed increase in potassium conductance? We suggest that it may do this by letting calcium ions leak in during the roughly 1–3 min long periods of membrane depolarization observed to follow puncture. The resulting increase in the intracellular calcium level would then be slowly reversed by the outward calcium pump [12]. The main basis for this hypothesis is the growing evidence that small increases in intracellular Ca^{2+} can greatly increase potassium conductance in a variety of cells [1, 3]. A slight extension of this hypothesis would attribute the reduction in membrane resistance which

persists after a return to sea water from 10 × K^+ sea water (fig. 2*b*) to a persistance of a high internal calcium level which was produced while the cell was in the high K^+ sea water.

The above discussion concerns the developing egg. The disturbance produced by puncture of the unfertilized egg has quite a different character. Unlike the developing egg, its membrane undergoes a largely irreversible depolarization upon second puncture. Whatever the mechanism of this may be it obviously suggests that the true, undisturbed value of the unfertilized egg's average membrane potential is larger (i.e. more negative) than the value of −33 mV recorded after first puncture.

Implications

Our new results indicate that the true membrane potential of the unfertilized *F. serratus* egg is somewhat more negative than −33 mV. This is considerably larger than the −19 mV value previously reported for *F. serratus* [2] or of −22 mV and −16 mV for *F. furcatus* and *P. fastigiata* respectively [12]. We believe the larger value to be closer to the true one. This upward revision of the best estimate for the unfertilized fucoid egg's membrane potential is similar to the conclusion of Ito & Yoshioka for the unfertilized sea urchin egg [5]. They reported that in some 'anomalous cases', unfertilized *Hemicentrotus* and *Temnopleurus* eggs exhibited membrane potentials up to about −40 mV, a value much higher than those generally reported for unfertilized echinoderm eggs, but closer in their opinion to the true value.

The irreversible depolarization produced by second puncture also suggests that the $1–2 \cdot 10^3$ ohm · cm² value for the resistance of the unfertilized *F. serratus* egg which is reported here, (as well as the very similar values previously reported for *P. fastigiata* [12]), are all serious underestimates. Confirmation of this is provided by the relatively reliable tracer flux method: It gives a value of about 10^4 ohm · cm² for the specific resistance of the unfertilized *P. fastigiata* egg membrane [10].

Despite the lasting disturbance produced by puncture, the membrane potential values previously reported for developing fucoid eggs seem reliable. However, the conductances and evidences of excitability obtained with intracellular micropipettes are seriously in error. Estimates of membrane resistance from ion flux measurements [10] and indicators of excitability from extracellular electrodes [7, 8] are clearly more reliable. Despite all this, our views of the slow electrical changes which accompany activation of the fucoid egg do not seem to require any qualitative revision. Activation involves large increases in resting potential, conductance and excitability produced in good part by an opening of potassium channels.

Anderson et al. have recently reported a remarkably similar course of impalement recovery in pea root cells [13]. As in *Fucus* eggs, the membrane potential recovers within 1 min after impalement, while the membrane conductance takes about 30 min to fall back to an apparently stable value of $1–2 \cdot 10^3$ ohm · cm². However, it should be noted that the membrane potential in this terrestrial plant is in good part electrogenic rather than diffusional (as in *Fucus*) so the exact explanation of the pea root results must necessarily differ. In fact, the authors suggest that the rapid recovery of the membrane potential in pea roots is effected by some rapid compensatory increase in the electrogenic current; rather than our suggestion for *Fucus* eggs of an injury that can hardly change the membrane potential since it only opens more potassium channels in a membrane whose

conductance is already dominated by such channels.

Finally, it may be worth noting that the relatively subtle and only slowly reversed puncture damage shown in our reinvestigation of a fucoid egg, may well be a serious source of error in intracellular recording from other inexcitable cells. Such cells may be relatively susceptible to such damage because they may not normally have to deal with inward leaks (via action potentials) of as much calcium, sodium etc., as do excitable cells like nerve and muscle.

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