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CHANGES IN SODIUM, CALCIUM AND POTASSIUM CURRENTS DURING EARLY EMBRYONIC DEVELOPMENT OF THE ASCIDIAN *BOLTENIA VILLOSA*

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SUMMARY

1. The whole-cell variation of the patch clamp was used to study ion channel properties in the unfertilized oocyte, and in surgically isolated blastomeres from 2-, 4-, and 8-cell embryos of the ascidian, *Boltenia villosa*.

2. The unfertilized oocyte has three major voltage-dependent currents: a transient, inward Na^+ current; a transient, inward Ca^{2+} current; and an inwardly rectifying K^+ current.

3. The total surface area of the embryo, either measured by capacitance or calculated from cell diameters, increased about 2·5-fold between fertilization and the 8-cell stage.

4. The Na^+ current almost completely disappeared from the embryo by the time of first cleavage and was undetectable in any of the blastomeres at the 8-cell stage. This loss was too large to be explained by the dilution of channels in the oocyte due to newly added membrane.

5. Both the Ca^{2+} current and the inwardly rectifying K^+ current were maintained at constant or slightly increased density through the first three cleavage cycles. This suggests that these channels are added along with new membrane during these stages.

6. No differences in mean current densities of blastomeres of different developmental fates were detected through the 8-cell stage.

7. Continuous recordings in single egg cells between fertilization and first cleavage, using two-microelectrode voltage clamp, revealed the increase in capacitance, Ca^{2+} current amplitude, and K^+ current amplitude, and the loss of Na^+ current predicted from the blastomere studies.

INTRODUCTION

Ascidians have been used extensively to study the determination of cell lineages in the early embryo because unlike most chordates, they show a pronounced mosaicism of development (Conklin, 1905; Reverberi, 1971). Cytoplasmic determinants segregate spatially in the embryo shortly after fertilization, and the various cell lineages of the larva are established when these determinants are partitioned by subsequent cleavages (Ortolani, 1958; Whittaker, 1979, 1982; Nishida & Satoh,

1983*a*,*b*; Jeffery & Meier, 1983). With one exception (Reverberi & Minganti, 1946), blastomeres isolated from early stages are capable of autonomous development into appropriate larval tissues, indicating that there are minimal inductive effects in the embryo (Reverberi, 1971; Whittaker, 1979; Whittaker, Ortolani, & Farinella-Ferruzza, 1977; Meedel & Whittaker, 1983).

Like the oocytes of many marine invertebrates (Hagiwara & Jaffe, 1979), ascidian oocytes are electrically excitable and possess populations of voltage-dependent ion channels similar to those found in mature excitable cells (Okamoto, Takahashi, & Yoshii, 1976; Thompson & Knier, 1983). Hence the ascidian has been used to investigate the ontogeny of electrical excitability between the oocyte and the larval stages (Takahashi, Miyazaki, & Kidokoro, 1971; Takahashi & Yoshii, 1981; Hirano & Takahashi, 1984; Hirano, Takahashi, & Yamashita, 1984). Previous studies on the development of ion channel properties in ascidian embryos have used two-microelectrode voltage clamp of embryos cleavage-arrested with cytochalasin B and in some cases on intact embryos during normal development (Takahashi & Yoshii, 1981; Hirano *et al.* 1984). Cleavage-arrested embryos undergo a complex sequence of changes in electrical properties corresponding to those which would have been required in the intact embryo to produce the diversity of excitability types found in larval tissues (Takahashi & Yoshii, 1981; Hirano & Takahashi, 1984; Hirano *et al.* 1984).

We have taken a different approach. We have isolated identified blastomeres surgically from early embryos at different stages and studied them using the whole-cell variation of the patch clamp technique (Hamill, Marty, Neher, Sakmann, & Sigworth, 1981). This approach offers two principal advantages. First, the normal cleavage cycles of the embryo are preserved, so that the effects of the addition of new membrane during cleavage cycles can be studied. Second, differences in electrical properties among blastomeres can be assessed in acutely isolated blastomeres, whereas in the intact embryo these might be masked by electrical coupling among different cell types (see Jaffe & Guerrier, 1981). We have used the ascidian *Boltenia villosa*, which has an orange pigment that segregates in the egg after fertilization and partitions differentially into blastomeres of different developmental fates as cleavage progresses (see Jeffery, 1982). This allows isolated blastomeres to be readily identified. Since the most intense pigmentation occurs in the muscle lineages, presumptive muscle blastomeres can be identified visually at any stage from the 4-cell embryo to the tadpole larva.

In this paper, we report the results of experiments on the unfertilized oocyte and the 1-, 2-, 4- and 8-cell embryos. We have studied all blastomeres at each of these stages under voltage clamp, and have analysed developmental changes in the Na^+, Ca^{2+}, and inwardly rectifying K^+ currents, the three principal voltage-dependent currents found before fertilization.

Some of these results have been presented in abstract form (Block & Moody, 1986).

METHODS

Specimens of the ascidian *Boltenia villosa* were collected from docks and pilings off San Juan Island, Washington. Animals were maintained at 10–12 °C in natural sea water, fed on algal suspensions, and kept on a constant light regime to prevent spawning. All experiments were done at 10–12 °C.

Preparation of gametes

Gonads (combined ovo-testes) were removed from the animals, minced in iced sea water, and rapidly washed with several hundred millilitres of 10–12 °C sea water, first through a 220 μm and then a 130 μm Nitex filter. The first filter retains gonadal debris, and the second retains oocytes but passes sperm. The eggs were than allowed to sit for 1 h in cold sea water to allow maturation and to expand the perivitelline space, which facilitates dechorionation. For fertilization, pairs of animals were dissected, and sperm collected either as the first wash through the 130 μm filter, or directly from the minced gonads. Eggs were fertilized with the heterologous sperm.

Eggs were dechorionated manually with electrolytically sharpened tungsten needles. No enzyme treatments were used, since such treatments have been shown to alter developmental fates in ascidian embryos (Ortolani, Patricolo & Mansueto, 1979). Since fertilization in *Boltenia* requires the chorion-follicle cell layer, fertilized eggs were dechorionated after fertilization was determined to be successful, as judged by the vegetal capping of orange pigment at about 15 min following sperm penetration. This was done for all embryo experiments, since dechorionation is difficult after first cleavage. Blastomeres were separated surgically, using tungsten needles; cells were separated after each cleavage, to increase the yield of healthy blastomeres and so that cells from the right and left halves of the same embryo could be studied at different stages. Blastomeres were identified by size, pigmentation, and position in the embryo.

Solutions

Artificial sea water had the following composition (mM): NaCl, 400; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 50; HEPES, 10; pH 8·1. The divalent composition of 50 mM-Sr^{2+} sea water was 0 mM-Ca^{2+}, 50 mM-Sr^{2+} and 10 mM-Mg^{2+}. The pipette solution for whole-cell recording contained (mM): KCl, 400; NaCl, 10; EGTA, 10; HEPES, 20; pH 7·3. Substitution of 90% of the Cl^- by aspartate, or decreasing the K^+ concentration to 200 mM did not alter the results. All solutions were filtered at 0·22 μm before use.

Electrical recordings

Most recordings were done using the whole-cell variation of the patch clamp technique (Hamill *et al.* 1981). Pipettes were pulled from borosilicate micropipette glass using a two-stage pull, to a tip diameter of 2–5 μm, and then fire polished immediately before use. Pipette resistances were 0·5–4 MΩ in sea water. The egg and blastomere membranes appear to be very clean after dechorionation, and using gentle suction we obtained seal resistances of 5–200 GΩ almost without fail. The whole-cell configuration was achieved by transient application of greater suction, and membrane rupture was monitored both visually as the entry of cytoplasmic yolk granules into the pipette tip, and electrically, as a large increase in capacitance. In the whole-cell mode, input resistances in unfertilized oocytes ranged from 100 MΩ to more than 5 GΩ, as judged by the slope of the steady-state current–voltage (I–V) relation in the potential range −60 to −40 mV, where the inward rectifier is not activated. All voltages were corrected for the junction potential between the pipette and bath solutions. Series resistance compensation was used in all experiments, and the adequacy of compensation was confirmed in a series of experiments which used combined microelectrode recording and suction pipette clamp. The low current densities present in the oocytes of ascidians and many other animals (see Hagiwara & Jaffe, 1979) allow this type of voltage clamp to be used successfully on such large cells. Most of our recordings were terminated after less than 15 min, and so no loss of currents or gradual shifts in apparent surface potential were noted (Byerly & Hagiwara, 1982; Fernandez, Fox & Krasne, 1984). Since the pipette diameter was small in relation to the cell, it was not surprising that the pipette solution had little effect on the currents recorded. See Lee, Akaike & Brown (1978) and Byerly & Moody (1986) for direct measurements of exchange rates of various ions during internal perfusion with large pipettes.

Isolated blastomeres were capable of at least several rounds of cleavages, in some cases while still attached to the suction pipette. In several cases, blastomeres were recovered from the experimental chamber after completion of the experiment, and allowed to develop. In about half of these cases, development into tadpoles (recovered at the 1-cell stage) or partial tadpoles (recovered blastomeres; see Whittaker, 1979) occurred. For two-microelectrode voltage clamp, electrode resistances were 8–15 MΩ in sea water. Electrodes were filled with 3 M-KCl. In most fertilized eggs, first cleavage occurred at the appropriate time even after microelectrodes had been in place for more than 1 h.

Data were recorded on FM magnetic tape, and on replay current records were filtered at 0·2–1·5 kHz using a filter with an 8-pole Bessel characteristic. Voltages were referenced to an Ag–AgCl ground. We found it necessary either to isolate the silver wire from the bath through a sea-water agar bridge, or to have continuous solution flow during the experiment, since silver ions appeared to block the Na^+ currents. Cell capacitance was measured by applying a 10 mV, 50 Hz triangle wave command to the voltage clamp amplifier, and measuring the amplitude of the resulting square-wave current signal (see Moody & Bosma, 1985).

RESULTS

Ionic currents in the unfertilized Boltenia oocyte

We have analysed the voltage-dependent ion currents in the unfertilized oocyte using both the two-microelectrode voltage clamp and the whole-cell suction pipette clamp (see Methods). Details of the two-electrode analysis are published elsewhere (Hice & Moody, 1987). Results of the whole-cell clamp are summarized in Fig. 1 and will be described briefly here.

Three principal voltage-dependent currents were seen under voltage clamp in the unfertilized oocyte.

(1) A transient inward Na^+ current was seen during depolarizing voltage clamp steps from holding potentials more negative than −60 mV (Fig. 1). Peak current was at −30 to −40 mV. Na^+ current amplitude, measured from holding potentials sufficiently negative to saturate h_∞ (−100 to −110 mV; Fig. 1*C*), ranged from 150 pA to 6 nA (see Fig. 3).

(2) A transient inward Ca^{2+} current was seen during depolarizations to voltages more positive than −30 mV (Fig. 1*A* and *B*). Ca^{2+} current inactivation was dependent on calcium entry rather than voltage (Hice & Moody, 1987; see Eckert & Chad, 1984). In normal external solutions (10 mM-Ca^{2+}) whole-cell Ca^{2+} currents ranged from zero to 300 pA. The Ca^{2+} current was best visualized by holding the cell at −50 or −60 mV to inactivate the normally much larger Na^+ current and using a high concentration of Sr^{2+} (50 mM), which is more permeant than Ca^{2+} in the channel, and does not permit inactivation, and which, unlike Ba^{2+}, does not block the inward rectifier (unpublished observation; see Hagiwara, Miyazaki, Moody & Patlak, 1978). Figure 5 shows the range of Ca^{2+} current densities obtained in unfertilized oocytes using 50 mM-Sr^{2+} external solution.

(3) An inwardly rectifying K^+ current was activated at voltages more negative than −70 mV. The range of current densities encountered in unfertilized oocytes is indicated in Fig. 7.

There was little, if any, outward current negative to +40 mV in most oocytes. The few cells which showed enough outward current to interfere with accurate Ca^{2+} current measurements (3 out of 59) were not included in our analysis of the Ca^{2+} current. Two components of the Ca^{2+} current were apparent in some cells when Ca^{2+} was used as the permeant ion (see also Hice & Moody, 1987).

The current did not inactivate fully during prolonged depolarizing voltage pulses, and depolarizing pre-pulses to near −30 mV could selectively eliminate the inactivating component. Since most blastomere experiments were done using Sr^{2+} as the permeant ion, we did not separate these

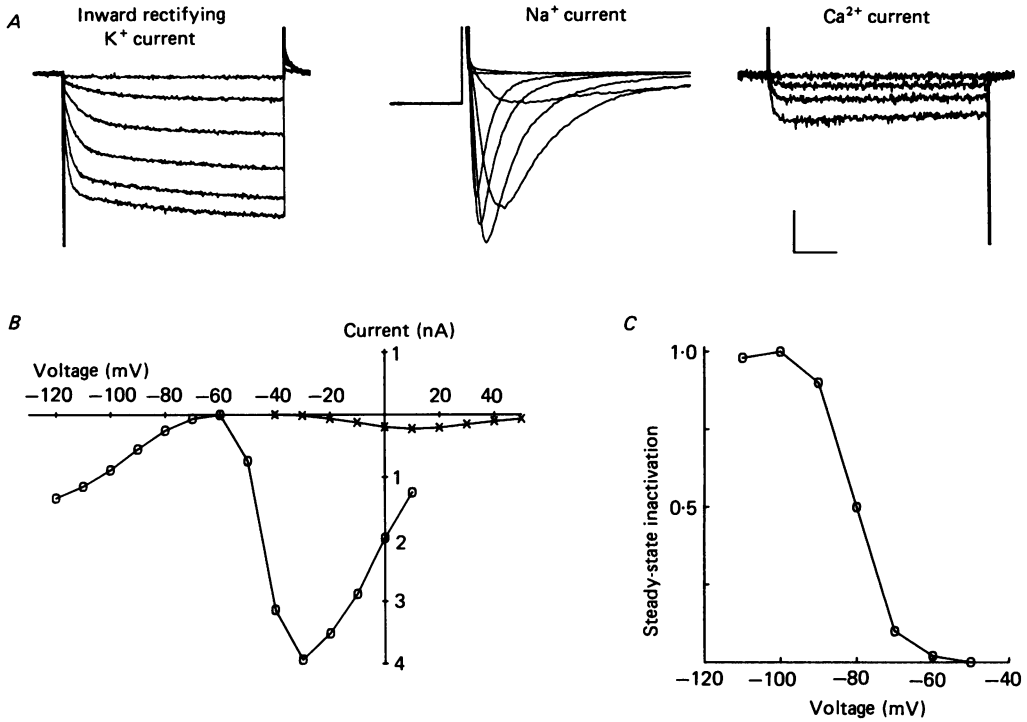

Fig. 1. *A*, voltage-clamp records of the inwardly rectifying K^+ current, the Na^+ current, and the Ca^{2+} current in the unfertilized oocyte. The following pulse paradigms were used. Inwardly rectifying K^+ current: holding potential, −60 mV, pulses to −70 through −120 mV in increments of 10 mV; Na^+ current: holding potential, −100 mV, pulses to −70 through −10 mV in increments of 10 mV; Ca^{2+} current: holding potential −60 mV, pulses to −30, −20, −10 and +10 mV. Calibration bars: 500 pA, 50 ms for K^+ currents; 1 nA, 20 ms for Na^+ currents; 200 pA, 50 ms for Ca^{2+} currents. The apparent leak in the Na^+ current records is due to the tonic activation of the inward rectifier at −100 mV. Ca^{2+} currents were recorded in 50 mM-Sr^{2+} external solution; other currents in standard artificial sea water. *B*, current–voltage relation for the unfertilized oocyte. The inwardly rectifying K^+ current, the Na^+ current (○), and the Ca^{2+} current (×) were taken from holding potentials as in *A*. Na^+ currents in this and all Figures were measured as the difference between peak and steady-state currents during a 400 ms voltage pulse. *C*, steady-state inactivation *vs.* voltage curve for the Na^+ current.

components further. We have also recorded in some cells a slow, Ba^{2+}-resistant component of inward rectification activated at potentials negative to −120 mV. Because it is not prominent in many cells, and is activated only at very negative potentials, we have not analysed this current further.

Results obtained with two-microelectrode and suction pipette techniques were very similar. Current–voltage relations and inactivation *vs.* voltage curves were similar in the two sets of measurements, as were current amplitudes and kinetics. The wide variation among cells in Na^+ current amplitudes was seen with both techniques.

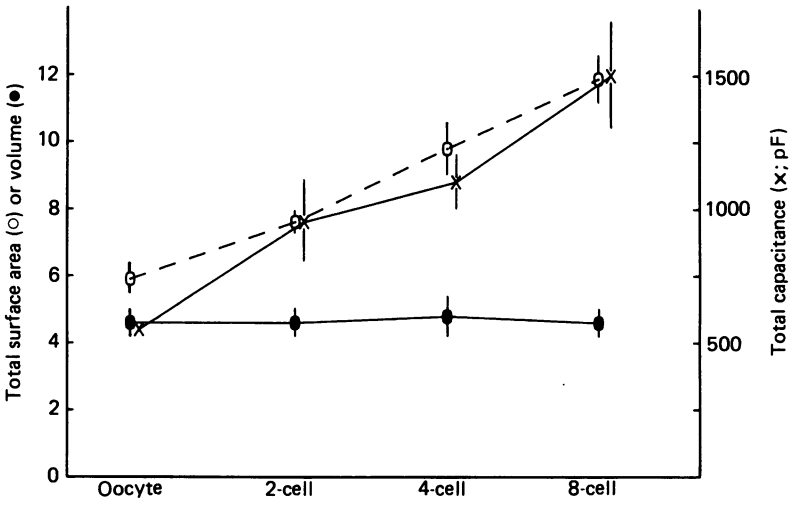

Fig. 2. Total embryo volume (●), total embryo surface area calculated from cell diameters (○), and total embryo capacitance (×) in the unfertilized oocyte and the 2-, 4- and 8-cell stages. See text for method of capacitance measurement. Surface area is expressed as $\mu m^2/4$; volume as $\mu m^3/(4/3)$. Standard deviations are shown as vertical bars.

Since the suction pipettes were small in relation to cell diameters, no time-dependent changes in currents were seen for recordings up to 1 h in duration. Capacitance values obtained in oocytes with the suction pipette clamp were about 20% less than those obtained using two microelectrodes, probably as a result of residual uncompensated series resistance. This error is small in relation to the developmental phenomena described below; its effect (which should decrease as the cells become smaller) would be to make the two estimates of oocyte surface area in Fig. 2 agree better, and to reduce the tendency of Ca^{2+} and K^+ current densities to increase in Figs 5 and 7.

Development of electrical properties through the 8-cell stage

The purpose of these experiments was to characterize ionic currents under voltage clamp in each blastomere at the 2-, 4- and 8-cell stages and compare them with currents found in the unfertilized oocyte. Two questions were of particular interest to us. First, what is the effect of new membrane addition during the first three cleavage cycles? Second, do 8-cell stage blastomeres of different developmental fates have different electrical properties? In this section, we have omitted the 1-cell fertilized stage, because the time between fertilization and first cleavage was sufficiently long for us to measure time-dependent changes in the currents in single cells, and these results are presented separately below.

Surface area, capacitance and volume changes. To quantify the amount of membrane addition during the first three cleavage cycles, we measured total surface area at each stage by determining total capacitance under voltage clamp. Total embryo capacitance was estimated from each blastomere studied by multiplying the cell capacitance by the number of blastomeres in the embryo from which the cell

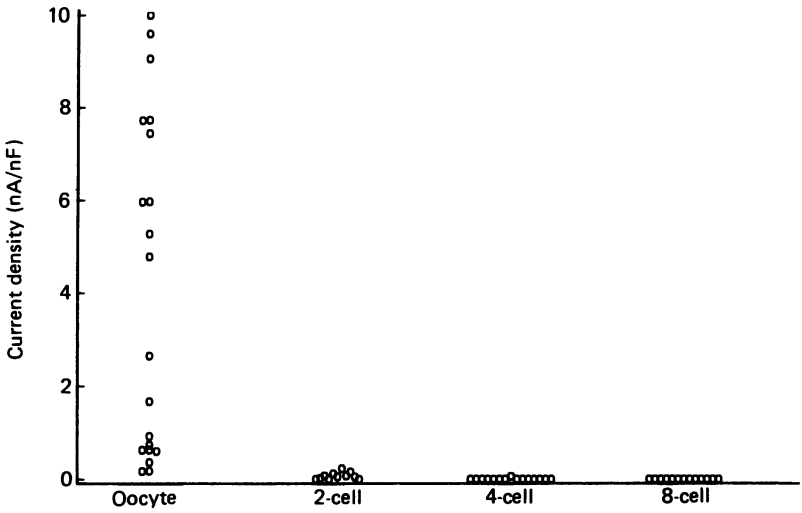

Fig. 3. Development of the Na^+ current. Each point represents the peak Na^+ current density in a single oocyte or blastomere from the stage indicated. Na^+ currents were measured from a holding potential sufficiently negative to remove inactivation (-100 or -110 mV). Na^+ currents in Figs 3 and 4 were measured in 50 mM-Sr^{2+} external solution, because the time between cleavages did not permit solution changes.

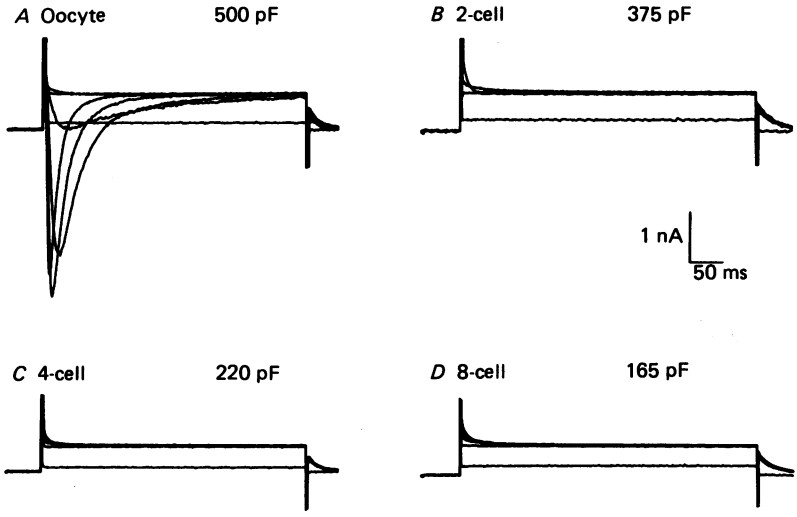

Fig. 4. Voltage clamp records of Na^+ currents from the unfertilized oocyte and individual blastomeres at the 2-, 4- and 8-cell stages. The capacitance for each cell is also given. Each panel shows currents recorded from a holding potential of -100 mV; steps are to -90, -70, -60, -50, -40, -30 and -20 mV, except in *B*, where the -50 and -20 mV steps are omitted for clarity. A very small Na^+ current is visible in *B*, none in *C* or *D*. The apparent broadening of the capacitative transient at the pulse onset in *B*, *C* and *D* is caused by the deactivation of the inward rectifier at voltages positive to -70 mV. The apparent leak is due to the tonic activation of the inward rectifier at -100 mV; note the superposition of steady-state currents for voltages positive to -70 mV, indicating minimal leakage current.

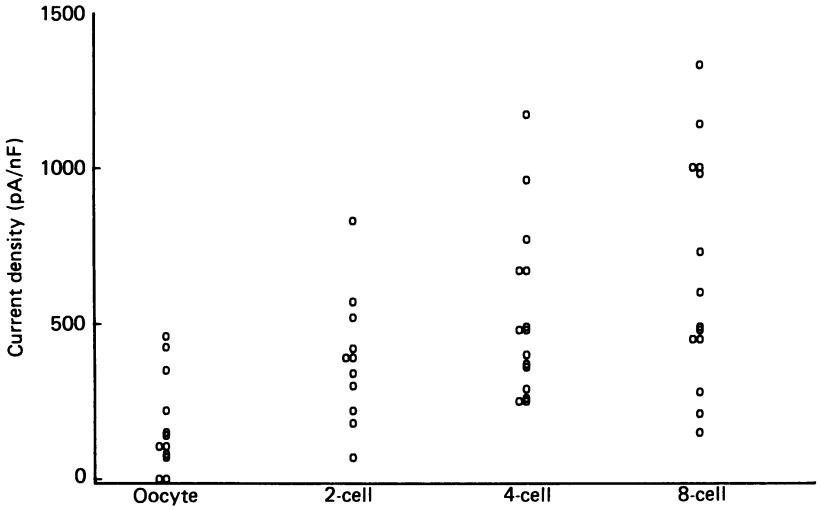

Fig. 5. Development of the Ca^{2+} current. Ca^{2+} current densities are plotted for the unfertilized oocyte and individual blastomeres as in Fig. 3. All currents were measured from a holding potential of −60 mV in 50 mM-Sr^{2+} external solution.

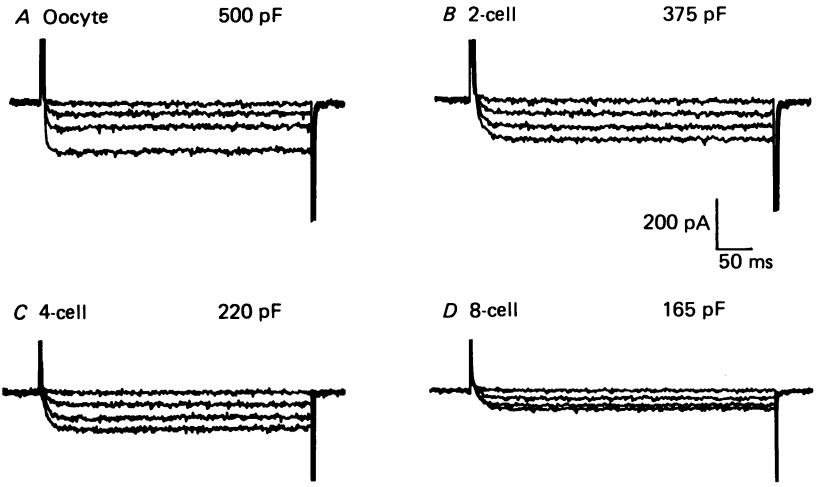

Fig. 6. Voltage-clamp records of Ca^{2+} current from the unfertilized oocyte and individual blastomeres at the 2-, 4- and 8-cell stages. All currents are taken from a holding potential of −60 mV in 50 mM-Sr^{2+} external solution. Voltage steps are to −30, −20, −10 and +10 mV. Capacitance values for each cell are also given. Note that the small peak current at the 8-cell stage (*D*) divided by the capacitance results in a current density similar to that in the oocyte and at the 2- and 4-cell stages.

came. Measuring total embryo capacitance directly by recording from all blastomeres of a single embryo was virtually impossible at the 4- and 8-cell stages, since the time between cleavages is short, and it is difficult to surgically isolate all blastomeres of a single embryo successfully.

These data are shown in Fig. 2, along with surface area and volume estimates based on cell diameter. Both estimates of surface area indicate an increase of

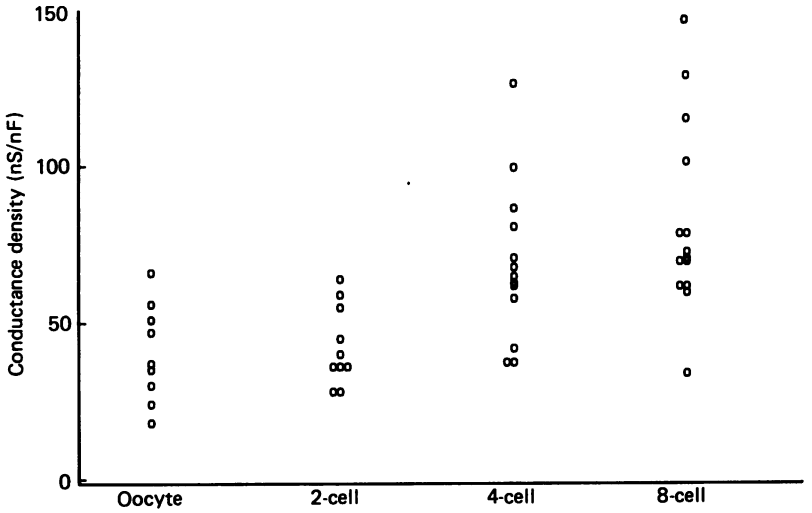

Fig. 7. Development of the inwardly rectifying K^+ current. Maximum slope conductance densities are plotted for unfertilized oocytes and individual blastomeres as in Figs 3 and 5.

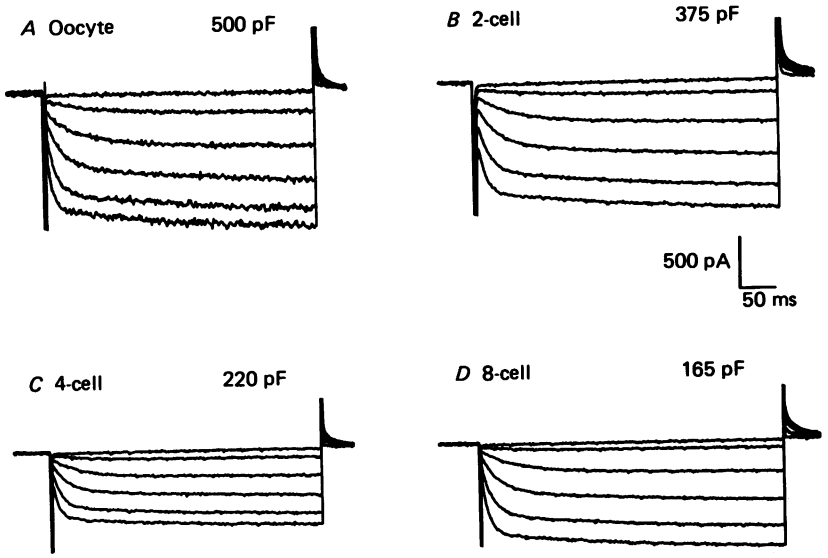

Fig. 8. Voltage clamp records of the inwardly rectifying K^+ current from the unfertilized oocyte and individual blastomeres at the 2-, 4- and 8-cell stages. Steps to −70 through −120 mV in increments of 10 mV from a holding potential of −60 mV are shown in each case.

approximately 2·5-fold between the unfertilized oocyte and 8-cell embryo. During this time total embryo volume, as expected, remains constant.

Na^+ current. The Na^+ current almost completely disappeared by the 2-cell stage, and remained absent through the 8-cell embryo in all blastomeres (Fig. 3). The unfertilized oocytes showed a great variability in Na^+ current density, which is

reflected in the scatter in the oocyte data in Fig. 3; the mean Na^+ current density in the unfertilized oocyte was about 3 nA/nF. By the 2-cell stage, all cells showed Na^+ current densities less than 0·3 nA/nF, with a mean of 0·1 nA/nF. At the 4-cell stage, only one cell out of fifteen had any detectable Na^+ current, and at the 8-cell stage, no Na^+ current was detected in any of the thirteen cells studied.

Figure 4 shows examples of voltage clamp records of Na^+ currents from each stage. In cells with no detectable Na^+ current, we used holding potentials at least as negative as −120 mV and sometimes −160 mV to ensure that the data could not be explained by a shift in the h_∞ curve. At the 2-cell stage, currents were often large enough to allow the current–voltage relation and h_∞ curve to be determined, and no changes from the oocyte parameters were seen. Estimates of kinetic parameters and reversal potential could not be made with reasonable accuracy with such small currents. However, in experiments described below, we were able to observe the loss of Na^+ current directly in single embryos between fertilization and first cleavage, and we did not observe changes in these parameters.

Ca^{2+} current. In contrast to the Na^+ current, Ca^{2+} current density remained constant during the first three cleavages (Fig. 5), the slight tendency to increase evident in Fig. 5 not being significant (two-tailed *t* test, oocyte *vs.* 8-cell data). If the oocyte Ca^{2+} current were diluted by newly added membrane, then mean density should be reduced by about 60% at the 8-cell stage (see Fig. 2). Data grouped by identified blastomeres showed no significant difference in Ca^{2+} current density between cells of different developmental fates at either the 4- or 8-cell stage. The reason for the increase in the variance of current densities within our sample at the 4- and 8-cell stages is not clear.

Figure 6 shows sample Ca^{2+} current records at each of the four stages. Since all Ca^{2+} current measurements were done in 50 mM-Sr^{2+} to increase the accuracy of the measurements, no inactivation was observed. However, since inactivation remains blocked in Sr^{2+}, we assume that inactivation remains Ca^{2+} dependent during these stages. The current–voltage relation of the Ca^{2+} current was similar in all blastomeres studied at each developmental stage.

Inwardly rectifying K^+ current. Results obtained with the inwardly rectifying K^+ current were similar to those for the Ca^{2+} current. A total of forty-six cells were studied, and the data are shown in Figs 7 and 8. Current density showed no significant change from the oocyte through the 8-cell blastomeres. No differences in the current–voltage relations were seen at various stages, and all blastomeres at each stage showed this current at the same mean density. As with the Ca^{2+} current, the variance in inward rectifier current densities increased at later stages.

Action potentials in oocytes and embryos. We carried out current-clamp experiments in a number of isolated blastomeres at each developmental stage in order to assess the effects of the changes described above on action potential waveform. All recordings were done in 50 mM-Sr^{2+} external solution to accentuate the effects of the Ca^{2+} current. The unfertilized oocyte responded to depolarizing current pulses with an action potential at a threshold of about −60 mV. The action potential has a rapidly rising phase which carries the potential to about 0 mV, followed by a long plateau or, at stronger stimulus intensities, a prolonged, slowly depolarizing phase (Fig. 9*A*). These two phases reflect the contributions of the Na^+

and Sr^{2+} currents, respectively. In 50 mM-Sr^{2+} solution, the action potential commonly lasted several seconds. At the 2-, 4- and 8-cell stages, blastomeres responded to depolarizing current pulses as expected from the selective elimination of the Na^+ current. As shown in Fig. 9*B–D*, blastomeres responded almost passively

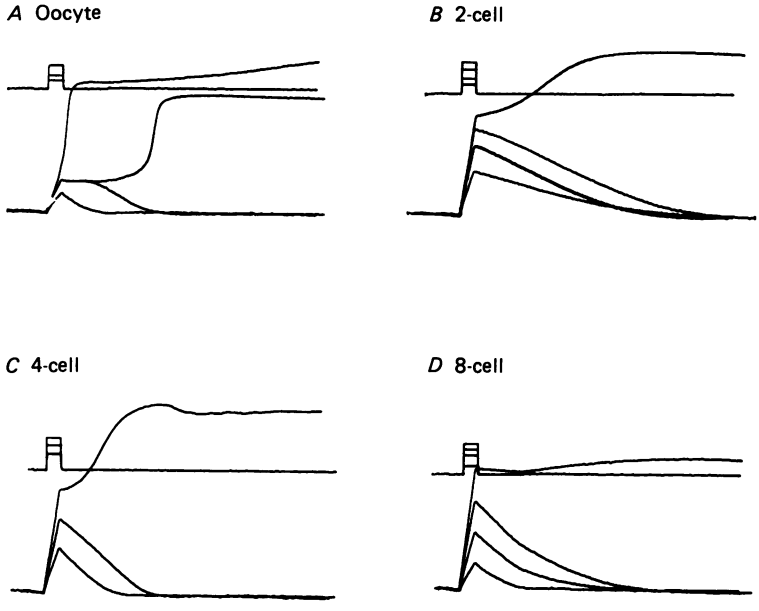

Fig. 9. Development of the action potential. Current-clamp records from the unfertilized oocyte and individual blastomeres from the 2-, 4- and 8-cell stages. Each cell was held at −80 mV with applied current, if necessary. The upper trace (current monitor) indicates bath potential. All records are taken in 50 mM-Sr^{2+} external solution. For calibration, the largest current pulse in *A* represents 50 pA, 20 mV, and is 50 ms in duration.

to depolarizations negative to about −20 mV. At about − 10 mV, a slow, prolonged depolarizing wave was seen, corresponding to the second phase of the oocyte action potential, mediated by the Sr^{2+} current through the Ca^{2+} channels.

Changes in ionic currents between fertilization and first cleavage

The results obtained in isolated blastomeres predict that between fertilization and first cleavage Na^+ current density should gradually decline to near zero, Ca^{2+} current and inward rectifier densities should remain constant or slightly increase, and capacitance should increase. We recorded such changes in nine fertilized eggs using a two-microelectrode voltage clamp to minimize the effects of dialysis by the suction pipette solution during the 120 min between fertilization and first cleavage. Control experiments confirmed the stability of currents during long-term recordings in unfertilized oocytes.

Figure 10 shows the results of one such experiment. Normalized current densities are plotted, so that no change in density with time implies an increase in current magnitude at the same rate as the increase in capacitance. The Na^+ current density

gradually declined to near zero, while at the same time both Ca^{2+} current and inwardly rectifying K^+ current densities increased. In some eggs, these densities remained constant. The data also show the gradual increase in capacitance as new

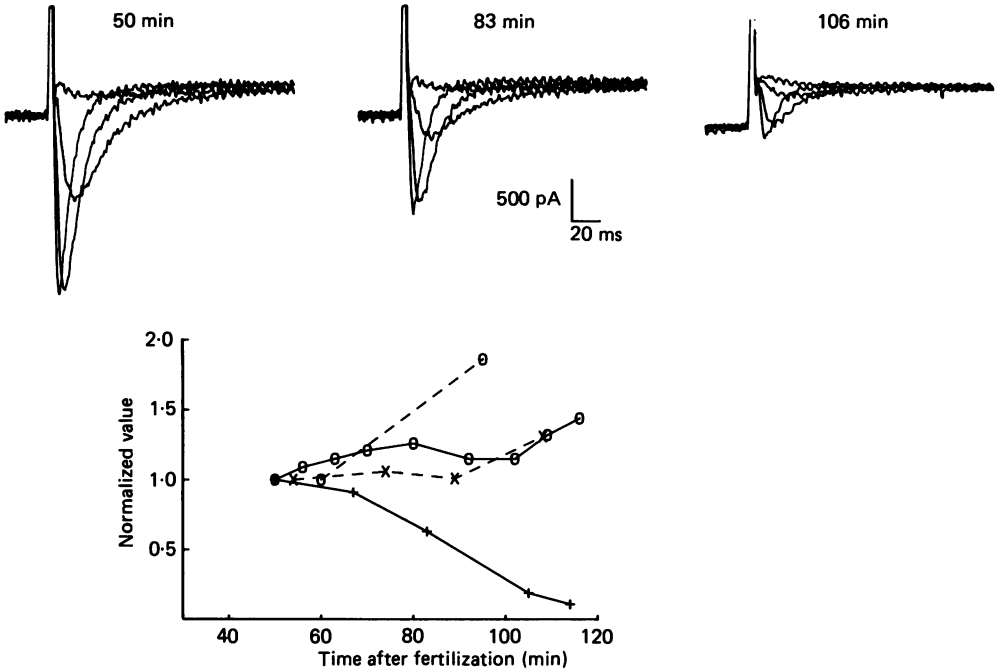

Fig. 10. Two-microelectrode voltage clamp records taken during a continuous impalement of a single fertilized egg from 50 min post-fertilization to the time of first cleavage (115 min). In the upper panels, Na^+ current records taken at the times indicated are shown. The holding potential was −100 mV, and steps to −60, −50, −40 and −30 mV are shown. The lower part of the Figure shows plots from the same cell of Na^+ current density (+), Ca^{2+} current density (○---○) inward rectifier density (×), and capacitance (○——○) *vs.* time after fertilization. All measurements are normalized to their initial values. Note that currents are all expressed as density, so that if current increased in parallel with capacitance, a horizontal line would result. All measurements were made in normal external solution, except for the Ca^{2+} current, which was measured in 50 mM-Sr^{2+}. The first records are at 50–60 min because the chorion must be removed, and the egg impaled, after fertilization. Based on measurements in unfertilized oocytes, normalized capacitance at time 0 would be approximately 0·77.

membrane is added in preparation for cleavage. The records in the upper part of Fig. 10 show sample Na^+ currents recorded at various times after fertilization. From analysis of such records, we concluded that there is no change in kinetics, voltage dependence, or estimated reversal potential of the Na^+ current during its disappearance. Thus the results obtained from single eggs between fertilization and first cleavage agree with the predictions of the blastomere data.

The earliest time points were obtained 40–50 min after fertilization because the chorion-follicle cell layer, which is required for fertilization in *Boltenia*, had to be removed to permit impalement. Although we have no information from these experiments about capacitance changes during the

first 50 min, the values obtained in unfertilized mature oocytes using the two-microelectrode clamped averaged 77% of the average value at 50–60 min after fertilization.

The transient decrease in capacitance beginning at about 90 min (30 min prior to cleavage) was seen consistently. We do not know the significance of this, but it is consistent with ultrastructural evidence of cyclical extension and retraction of microvilli associated with cleavage cycles reported in other ascidians (Satoh & Deno, 1984).

DISCUSSION

We have described three principal voltage-dependent ion currents in unfertilized oocytes of the ascidian *Boltenia villosa* using the suction pipette voltage clamp technique (see Hice & Moody, 1987, for a full description of the currents using the two-electrode voltage clamp), and have followed the development of these currents during early embryogenesis. The Na^+ current almost completely disappeared by the time of first cleavage. In contrast, both the Ca^{2+} current and the inwardly rectifying K^+ currents gradually increased in amplitude throughout the first three cleavage cycles, so that in spite of the 2·5-fold increase in total embryo surface area, their current densities in the 8-cell stage blastomeres remained equal to, or perhaps slightly greater than, their densities in the unfertilized oocyte. In all of these cases these changes were in current densities but not kinetics or voltage dependence.

There are differences between the developmental changes in the Na^+ and Ca^{2+} currents that we have found in *Boltenia* and those in *Halocynthia* studied by Takahashi and his colleagues (Takahashi & Yoshii, 1981; Hirano & Takahashi, 1984; Hirano *et al.* 1984). Our experimental approach differs from theirs. We used the suction pipette clamp on surgically isolated blastomeres, whereas they used two-microelectrode clamp of intact embryos at early stages (thereby recording from multiple blastomeres simultaneously), and, at later stages, embryos cleavage-arrested with cytochalasin B early in embryogenesis and then allowed to develop. In *Halocynthia*, they observed only about a 50% loss of the total embryo Na^+ current by the 8-cell stage and at later stages the loss never exceeded 75% (Takahashi & Yoshii, 1981, their Figs 3 and 4). In *Boltenia*, the Na^+ current decreased by more than 90% by the 2-cell stage, and was undetectable in the 8-cell embryo. This difference is unlikely to be due to methods used, since we found more than 90% loss of Na^+ currents before first cleavage also using two-microelectrode clamp (Fig. 10). Often in those experiments we continued the recordings through first cleavage, and once through the 4-cell stage, with similar results.

In *Boltenia*, the Ca^{2+} current increased steadily throughout the first three cleavage cycles, so that current density remained constant. In *Halocynthia*, the Ca^{2+} current virtually disappeared by the 8-cell stage (Takahashi & Yoshii, 1981). The *Boltenia* and *Halocynthia* Ca^{2+} channels, although similar in voltage dependence, differ in both their permeability sequences and inactivation mechanisms. The developmental fates of these two Ca^{2+} channel types might reflect their different sensitivities to modulation by cytoplasmic biochemical events. Such differences have been demonstrated directly in Ca^{2+} channels of mature neurones using the suction pipette technique (Fedulova, Kostyuk & Veselovsky, 1985; Matteson & Armstrong, 1986). We have begun a comparative study of various ascidian oocytes in our geographical

area (R. E. Hice, in preparation), and have found striking variability between species in the properties of Ca^{2+} channels. We plan to exploit these interspecies differences to investigate the developmental fates of distinct Ca^{2+} channel types.

The loss of Na^+ current between fertilization and first cleavage is a complex series of at least two separable events. A rapid loss of roughly 25% of the oocyte Na^+ current occurs within 30 min after fertilization (Hice & Moody, 1987). This loss is restricted to the vegetal hemisphere, and thus represents a 50% loss of Na^+ current in that half of the egg. This early loss is followed by, and is separable from, the almost total loss of Na^+ current from all regions of the egg, which is shown in Fig. 10. At present we have little information about the mechanism of either phase of Na^+ current loss. The spatial restriction of the early loss seems to be due to factors intrinsic to the egg, rather than being determined by the site of sperm entry, since fertilized animal-half egg fragments do not show the loss (Hice & Moody, 1987). Either phase of Na^+ current loss could be due to masking or block of channels by cytoplasmic constituents, or by physical removal of channels from the membrane. We are at present investigating these questions further.

Since the gradual increase in both Ca^{2+} and inwardly rectifying K^+ currents roughly parallels the addition of new membrane, it is logical to assume that newly added membrane contains these channels in about the same density as the pre-existing oocyte membrane. However, in *Halocynthia* the inwardly rectifying K^+ current increased gradually in amplitude through the 8-cell stage, even in cleavage-arrested embryos where minimal addition of new membrane occurs (see Takahashi & Yoshii, 1981). This raises the possibility that the maintenance of inward rectifier current density in our experiments in the face of substantial membrane addition results from a mechanism other than the simple addition of channels along with new membrane.

Differential behaviour of ion channel populations during periods of membrane addition or removal is also seen in other oocytes. In the starfish *Leptasterias*, the magnitude of the A-current closely parallels the total surface area both during oogenesis and meiotic maturation (Moody, 1985; Moody & Bosma, 1985). In constrast, the Ca^{2+} current in *Leptasterias* appears late in oogenesis, after cell growth has been completed, and fails in decrease during maturation, even in the face of a 50% loss of surface membrane over the course of 1 h.

We found no differences in the Ca^{2+} and inwardly rectifying K^+ currents among blastomeres of different developmental fates. This is not surprising, since these two channel populations are probably widely distributed among cells of the tadpole (Mackie & Bone, 1976; Hirano *et al.* 1984), and all major lineages are not separate in the ascidian embryo until the 64-cell stage (Nishida & Satoh, 1983*a*,*b*). With nerve and muscle lineages separate at the 8-cell stage, we expected to find muscle-lineage blastomeres lacking Na^+ channels, since tadpole muscle generates a purely Ca^{2+}-dependent action potential (Takahashi *et al.* 1971). However, all blastomeres at the 8-cell stage lack functional Na^+ channels.

Voltage clamping blastomeres isolated from later embryos will allow us to determine when differentiation of cell properties occurs among blastomeres of different lineages. Intracellular dialysis of potential modulators of channel function may clarify possible mechanisms of these early developmental events.

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