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Differential Developmental Fates of the Two Calcium Currents in Early Embryos of the Ascidian *Ciona intestinalis*

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Abstract. Two voltage-dependent calcium currents have been described in unfertilized eggs of the ascidian *Ciona intestinalis:* a low threshold, slowly activating current, and a high threshold fast one. According to the classical criteria for classification of calcium currents, they both share some of the features of L-like and T-like currents. We have studied these two calcium currents further by measuring their sensitivity to permeant ions, temperature and inhibitors. Both currents were sensitive to relatively high concentrations of nitrendipine, which was a selective blocker of the low threshold channel. The lanthanide ion gadolinium was a potent blocker of the low threshold current, and cadmium preferentially inhibited the high threshold current. The two calcium currents were regulated in a different manner after fertilization. The density of the high threshold current remained relatively constant, while the low threshold current was lost by the time of first cleavage. This loss following fertilization is similar to the loss of a low threshold sodium current in fertilized eggs of the ascidian *Boltenia villosa.* Block of the cell cycle with various compounds did not prevent loss of the low threshold calcium current. This observation adds weight to the hypothesis that a loss of excitability is a general property of early development. We conclude that fertilization can differentially modulate channel populations before first cleavage. The mechanism by which this occurs in the ascidian embryo has yet to be discovered.

Key words: Oocyte — Tunicate — Development — Patch clamp — Gadolinium — Nitrendipine

Introduction

The diversity of calcium channel types and their possible coexistence in the same cell are well established. Several papers and reviews have tried to place calcium currents into well-defined families according to their features, starting with works on marine animal oocytes (Hagiwara, Ozawa & Sand, 1975), followed by investigations on heart cells (Nilius et al., 1985) and sensory neurons (Nowycky, Fox & Tsien, 1985), and quoted in the widespread reference books (Hille, 1991; Nowycky, 1991). The two voltage-operated calcium channels of unfertilized oocytes of the tunicate *Ciona intestinalis* have been described and characterized at the single channel level (Bosma & Moody, 1990). They do not fit neatly into any described type: e.g. the high threshold activated current (classical signature for the L-like channel) has fast transient activation reminiscent of T-like behavior. To further characterize these oocyte calcium currents, we have measured the ion selectivity (calcium *vs.* barium), the effects of temperature, and the sensitivity of the currents to blockers (gadolinium and cadmium). Both currents were inhibited by a dihydropyridine at relatively high doses if compared to the efficiency of these compounds on mammalian cells.

We have determined that the functional expression of the calcium currents is regulated during development. Following fertilization, the low threshold current disappears while the high threshold current is maintained at constant density. The disappearance of the low threshold calcium current in *Ciona* oocyte following fertilization is similar to the loss of a low threshold activated sodium current present before fertilization in the ascidian *Boltenia villosa* and absent at the time of first cleavage (Block & Moody, 1987). The elimination of channel populations at specific developmental stages is not restricted to early ascidian embryos, and has been reported during terminal differentiation of several tissues: nerve (Kubo, 1989), muscle (Beam & Knudson, 1988) and glia (Sontheimer et al., 1989). In spite of this apparent generality, the mechanisms involved in selectively eliminating those currents could be different, and the nature of these mechanisms has yet to be addressed.

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Materials and Methods

Specimens of the ascidian *C. intestinalis* were collected at Thau, near Sète on the French Mediterranean coast or delivered by the Marine Biological Station of Roscoff (Brittany, France) and kept in a tank of cooled, circulating seawater at a constant light level. Sperm were drawn up directly from the spermiduct, and mature eggs were extracted from the oviduct. The chorion was manually removed from eggs with fine sharpened tungsten needles. The dechorionated eggs were then stuck to the bottom of a chamber made from a glass slide. Fertilization or self-fertilization was induced on dechorionated eggs. The starting time of fertilization was monitored by the associated change in electrical current under voltage clamp (Kozuka & Takahashi, 1982). The eggs were continuously perfused with a bath solution maintained at constant temperature (between 12 and 17°C, otherwise specified).

Solutions

Artificial seawater (ASW) contained (in mM): 400 NaCl, 10 KCl, 10 $CaCl_2$, 50 $MgCl_2$, 10 HEPES (*N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid)) pH 8.0. When barium was substituted for calcium, or when the external calcium concentration was changed, the magnesium concentration was adjusted to maintain total divalent concentration at 60 mM. Pipette solution contained (in mM): 200 KCl, 10 NaCl, 1 $MgCl_2$, 1 EGTA (ethyleneglycol-*bis*(B-aminoethyl ether) *N,N,N',N'*-tetraacetic acid), 20 HEPES pH 7.3. Nitrendipine (Sigma) was prepared as a 20 mM stock solution in ethanol, dissolved to final concentration and kept in the dark throughout the experiment. $GaCl_3$ (up to 20 μM) or $CdCl_2$ (up to 1 mM) was added to barium artificial seawater (50 Ba-ASW). Emetine, TLCK (Nα-*p*-Tosyl-L-Lysine Chloro-Methyl Ketone) and 6-DMAP (6-dimethylaminopurine) were obtained from Sigma and dissolved at their final concentration in artificial seawater.

Electrophysiological Recordings

Pipettes were pulled to resistances of 2–4 MΩ with a Mecanex BB-CH puller and never fire-polished. Eggs were patch-clamped in the whole-cell configuration using a BioLogic RK300 amplifier and pCLAMP software (Axon Instruments). Series resistance was compensated electronically, and was typically in the range of 3–6 MΩ. Long-term recordings could be done on the same cell for hours. No leak subtraction was done on any records. To estimate the cell surface area, membrane capacitance was measured by steps of current induced under triangle-wave voltage command (Moody & Bosma, 1985).

Results

The Two Calcium Currents of the Unfertilized Oocyte

In oocytes of *C. intestinalis,* two inward calcium currents stimulated by depolarization have been described (Bosma & Moody, 1990; Dale, Talevi & DeFelice, 1991). These calcium currents are shown in Fig. 1, both recorded at 13°C. The oocyte was held at a membrane potential of −80 mV in a bathing solution of either high calcium artificial seawater (*A1, B1*) or barium-substituted artificial seawater (*A2, B2*). The low threshold current peaks at voltages near −20 mV with activation times greater than 100 msec. The high threshold current peaks in about 20 msec for voltages between +20 and +30 mV. At higher voltages than +60 mV, we recorded (*not shown*) the outward current already described (Bosma & Moody, 1990), corresponding to efflux of potassium through calcium channels. In recordings in calcium (traces *A1* and *B1*), the inward rectifier potassium current is activated, and a negative current is present at the holding potential of −80 mV. This inward rectifier current is blocked by barium (traces *A2* and *B2*). When barium replaces calcium (*A2, B2*), the peak amplitude of the low threshold current is increased. This behavior is illustrated by the *I-V* curves of Fig. 1*C*. The kinetics of both currents were rather insensitive to substitution of calcium for barium. The dependence of the inward current peak amplitudes upon barium external concentration is illustrated in Fig. 2 for one oocyte, where the low threshold current was increased by a factor of 7 between 0 and 50 mM barium. The high threshold peak current was only slightly sensitive to barium substitution.

The absolute and relative peak amplitudes of the two calcium currents vary between different oocytes, as previously described. We agree with the conclusion of Bosma and Moody (1990) that there is no correlation between the amplitudes of the two currents. The variation in current amplitude in different oocytes does not correlate with the geographical origin (Atlantic or Mediterranean) of the animals. Changes in temperature had different effects on the two currents, as illustrated by Fig. 3. As expected, increasing temperature speeds the activation of both currents. Decreasing temperature helps their separation. For a 10°C increase in temperature, the peak amplitude of the high threshold current doubles, while the peak amplitude of the low threshold current is unaffected by the temperature change (Fig. 3*C*).

Selective Inhibitions

We provided additional characterization of the two calcium currents by investigating their inhibition by various compounds that differentially block each current. Cadmium, in the mM range, preferentially inhibits the high threshold current, as shown in Fig. 4. In contrast, gadolinium at low concentrations (in the μM range) selectively inhibits the low threshold current (Fig. 5). The trials for cadmium ($n = 6$) and for gadolinium ($n = 4$) were performed from a holding potential of −80 mV, and in a bath solution with 50 mM barium. In all these assays, before application of blockers, the low threshold and high threshold currents had similar am-

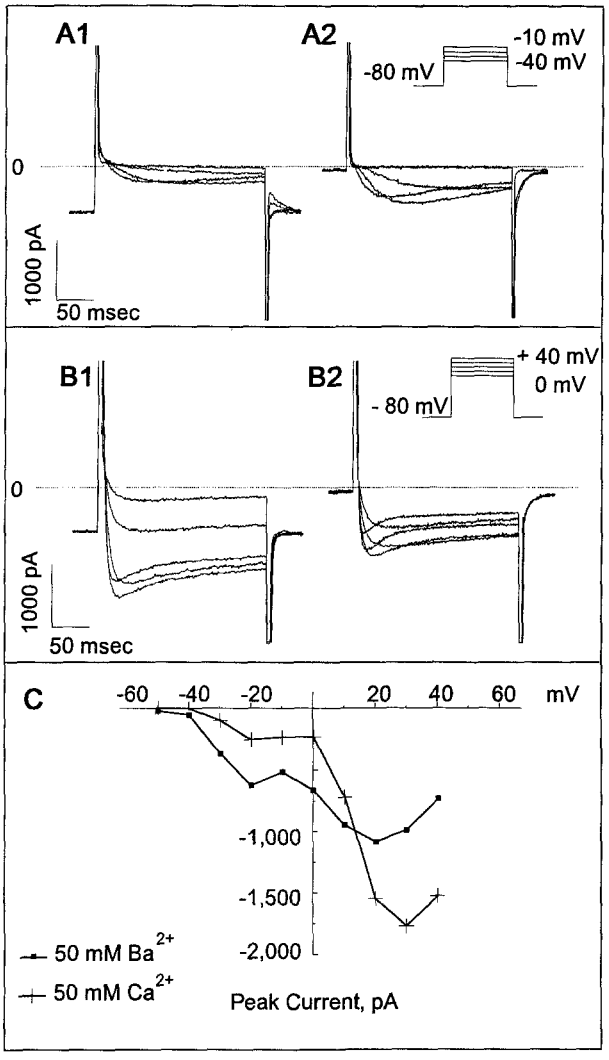

Fig. 1. The two calcium currents in unfertilized eggs of *C. intestinalis*. Holding potential clamped at −80 mV. (*A*) Low threshold currents induced by voltage pulses between −40 and −10 mV. (*B*) High threshold currents on the same egg induced by following pulses of voltage between 0 and +40 mV. (*A1*) and (*B1*) records in 50 Ca-ASW. (*A2*) and (*B2*) records in 50 Ba-ASW for the same egg. (*C*) Current-voltage relation in two different solutions: 50 Ca-ASW (+) and 50 Ba-ASW (■). Temperature was 13°C.

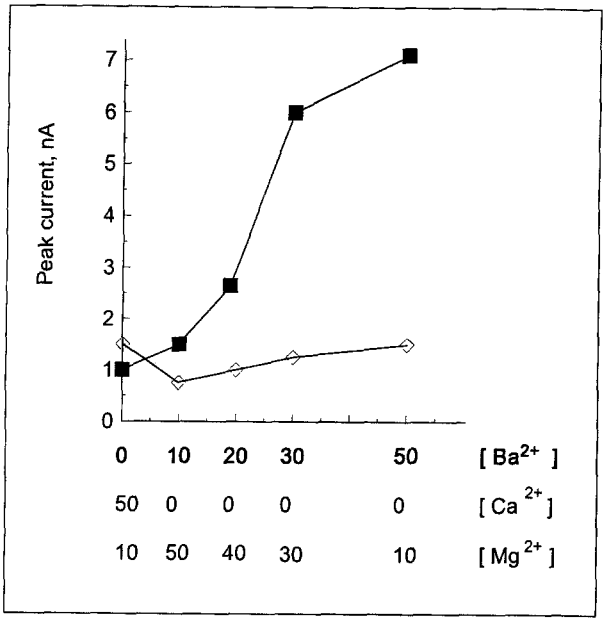

Fig. 2. Differential effect of barium substitution for calcium. Experiment on one egg from Mediterranean origin. Holding potential was −80 mV, and temperature 13.5°C. Concentrations of divalent ions in ASW are indicated under the plot. (■) Low threshold current. (◇) High threshold current.

plitudes. In these circumstances, the two currents were separated satisfactorily based on difference in activation threshold. Their relative inhibition was evaluated from the current amplitudes. The *I-V* plot of Fig. 5 (after block by gadolinium) shows that there is no contamination of the current at −20 mV due to the high threshold current. On the opposite, according to the *I-V* plot of Fig. 4 (after block by cadmium), the low threshold current could account for a small part of the current at +20 mV. Gadolinium in the same μM range of concentrations has been reported to inhibit calcium currents of neurons, myocytes and endocrine cells (Docherty, 1988; Biagi & Enyeart, 1990; Boland, Brown & Dingledine, 1991). It preferentially blocks the low threshold voltage-activated calcium current of *Ciona* oocyte, while in endocrine cells of the anterior pituitary, both low and high threshold calcium channels are equally inhibited (Biagi & Enyeart, 1990).

Since *Ciona* oocyte calcium currents share some characteristics with L-like currents, we tested whether dihydropyridines blocked either current. Such an experiment is shown in Fig. 6. We attempted to increase drug affinity for the channel according to the prescription by Bean (1984), and clamped the holding potential at positive voltage compared to the resting potential. This protocol had only a little effect of increasing the drug sensitivity on *Ciona* oocytes. In the five tested oocytes, both currents were sensitive to block by nitrendipine, with a preferential block for the low threshold current. The nitrendipine concentration required to completely suppress the low threshold current, e.g., 100 μM, was always high (when compared to the reported values recorded in numerous mammalian cells at higher temperatures) and never succeeded in totally eliminating the high threshold component (e.g., inhibition rate of 40%). The inhibitory effect of nitrendipine was not instantaneous, but occurred over minutes and was reversible, with a recovery kinetics slower than the inhibition kinetics (Fig. 6*A*). The different behavior of the three inhibitors is summarized by the plots of Fig. 7, showing that gadolinium, at concentrations lower than

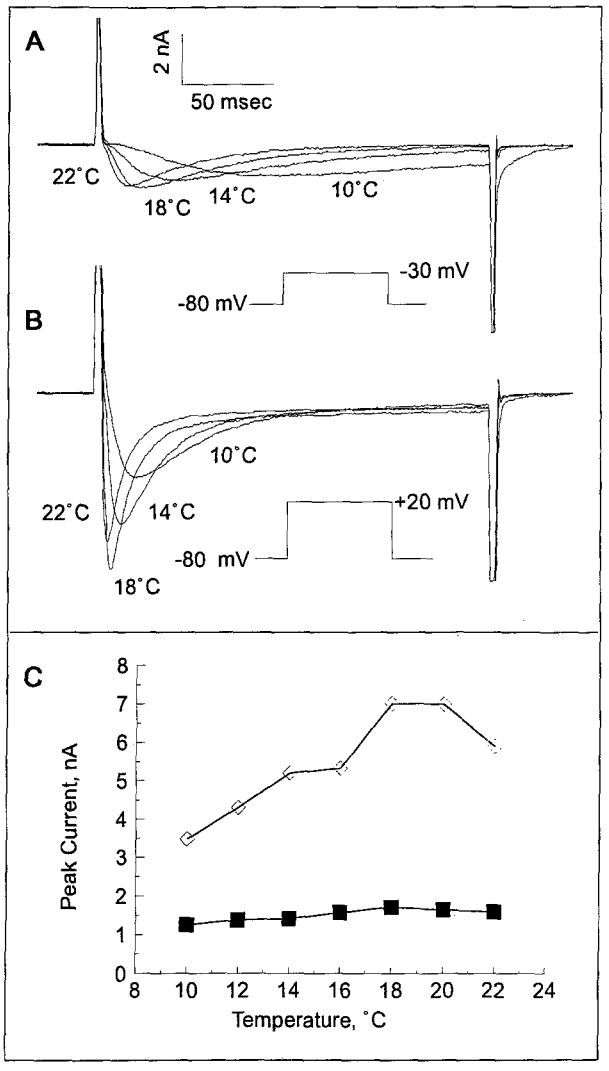

Fig. 3. Effect of temperature on calcium currents. All records were obtained in 50 Ba-ASW. (*A*) Low threshold current recorded at −30 mV from a holding potential of −80 mV. (*B*) High threshold current recorded at +20 mV from a holding potential of −80 mV. (*C*) Peak current-temperature relation. (■) Low threshold current. (◇) High threshold current.

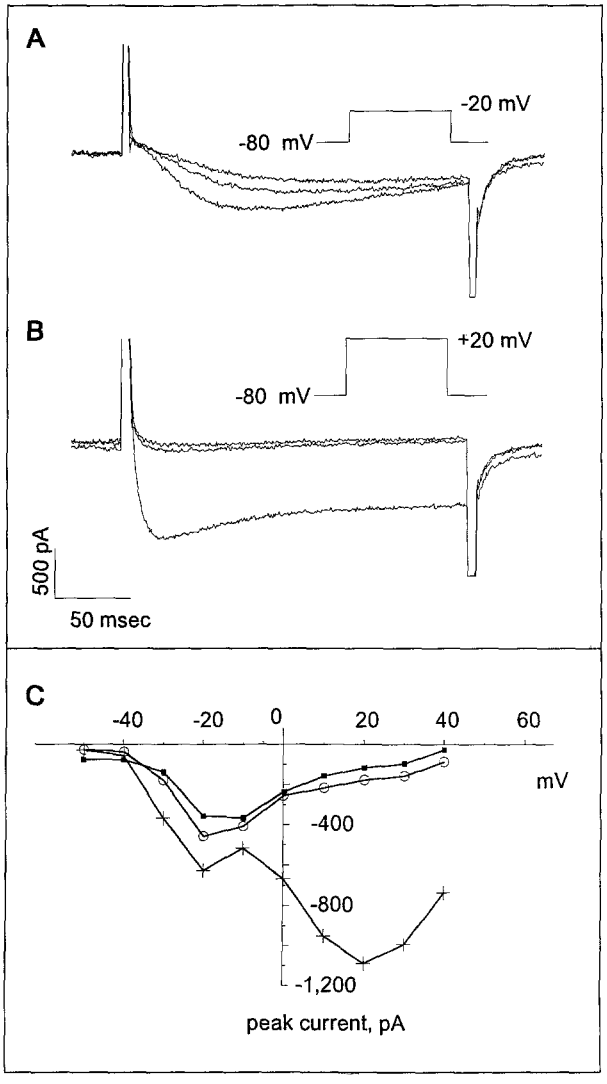

Fig. 4. Effect of cadmium on calcium currents. The two calcium currents were recorded from the same egg with an external solution of 50 Ba-ASW. Holding potential of −80 mV and temperature of 13°C. (*A*) Low threshold currents recorded at −20 mV sequentially in three different concentrations of cadmium, 0.0, 0.5 and 1 mM, from bottom to top on traces. (*B*) High threshold current recorded at +20 mV in the presence of the same three concentrations of cadmium. (*C*) Corresponding current-voltage relation: no cadmium (+), 0.5 mM (○) and 1 mM (■) cadmium.

5 μM, and nitrendipine are selective blockers of the low threshold channel.

Loss of the Low Threshold Current after Fertilization

We measured the behavior of the two calcium currents before and after fertilization, using long-term recordings from individual eggs. In unfertilized eggs, the currents were remarkably stable, as illustrated in Fig. 8, where each calcium current corresponds to 1 of 12 experiments conducted before fertilization. To examine currents following fertilization, we began monitoring the two calcium currents as soon as the current associated with the fertilization potential had declined. In some eggs, at early times after fertilization, the low threshold calcium current amplitude increased compared to its prefertilization level, a result similar to that observed for low threshold sodium currents of other ascidians (Kozuka & Takahashi, 1982; Coombs, Villaz & Moody, 1992). In all cases, the low threshold calcium current, measured every 5, 10 or 20 min, decreased until it could not be detected by the time of first mitosis. Unlike the

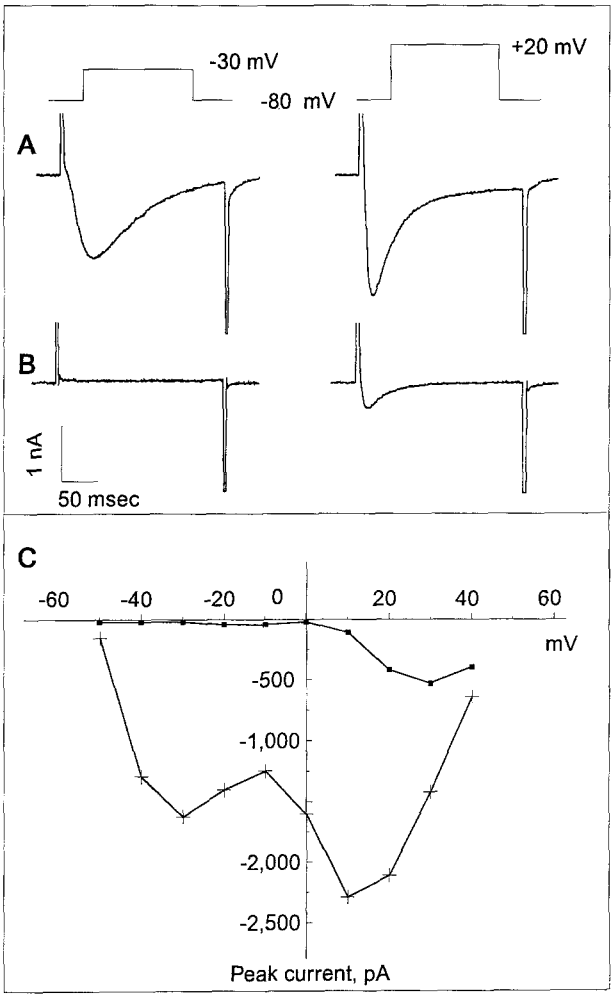

Fig. 5. Effects of 20 μM gadolinium on calcium currents. (*A*) The two calcium currents were recorded respectively at −30 mV (low threshold) and +20 mV (high threshold), from a holding potential of −80 mV at 14°C. (*B*) The two calcium currents recorded using the same stimulation paradigm in the presence of 20 μM gadolinium. (*C*) Corresponding current-voltage relation: no gadolinium (+), 20 μM gadolinium (■).

low threshold current, the high threshold calcium current was maintained at approximately the same amplitude during completion of meiosis and first cleavage (*see* Fig. 9). As judged by comparing the *I-V* plots for before and long after fertilization (Fig. 9*C*), the high threshold current does not change in voltage dependence of activation. Since changes in membrane surface area are expected to occur in that time (Coombs et al., 1992), we measured electrically the capacitance of the egg membrane as an indicator of the surface area (*data not shown*). We divided the peak currents, such as shown on Fig. 9*A* and *B*, by the capacitance at their recording time, and the resulting plot of the variations of current densities is shown in Fig. 10*B*. In the experiments corresponding to the data of Fig. 10*B* (as well as for Fig. 9), we chose to keep the egg in normal artificial seawater as much as possible; we transiently applied barium at recording times, and the interval of time between two protocols was around 20 min. The density of the high threshold current was maintained roughly constant and the density of the low threshold current decreased. The kinetics of decline recorded for all the experiments done, between 12 and 17°C, have not been satisfactory reproducible. On the eight recordings done for at least 1 hr (as shown for one oocyte in Fig. 10*B*), the half loss was obtained for five eggs within 20 min after fertilization. For the three others, the loss was only 20% in the first 20 min, and looked biphasic. In spite of this unexplained variability in eight recordings, in every one of the 10 eggs sampled around the time of first cleavage, the low threshold calcium current was always completely lost (Fig. 10*A*).

We tried to prevent the disappearance of the low threshold calcium current after meiosis resumption by compounds known to disturb cell cycle. We used three molecules known to act from the outside: emetine inhibits protein synthesis and thus cyclin production, while TLCK, a trypsin-like serine protease inhibitor, and 6-DMAP, a kinase inhibitor in the ATP-dependent pathway of cyclin destruction, are supposed to prevent cyclin degradation (Luca & Ruderman, 1989). Emetine does hinder polar body extrusion and cycling in cell surface area of the ascidians *B. villosa* (Coombs et al., 1992) and *C. intestinalis* (*data not shown*). In nine experiments with emetine (100 μM) applied at the time of fertilization on *Ciona* oocytes, the low threshold calcium current was still lost at the time or before the time of first division. An example of variation of egg capacitance and calcium current amplitudes in the presence of emetine is shown in Fig. 11. The low threshold current density declines while the high threshold current amplitude follows the increase in membrane capacitance, in spite of the protein synthesis inhibitor. Two fertilized eggs incubated in TLCK (300 μg/ml) and three fertilized eggs incubated in 6-DMAP (500 μM) were unable to proceed to first cleavage. Nevertheless, the loss of the low threshold calcium current occurred normally by the time of predicted first cleavage. Thus, none of the three, cell cycle perturbing compounds applied after fertilization was able to prevent the current loss.

Discussion

In this study, we present additional data to further characterize the two calcium currents activated by depolarization of the oocyte membrane of the ascidian *C. intestinalis*. Each of the calcium currents has a mixture

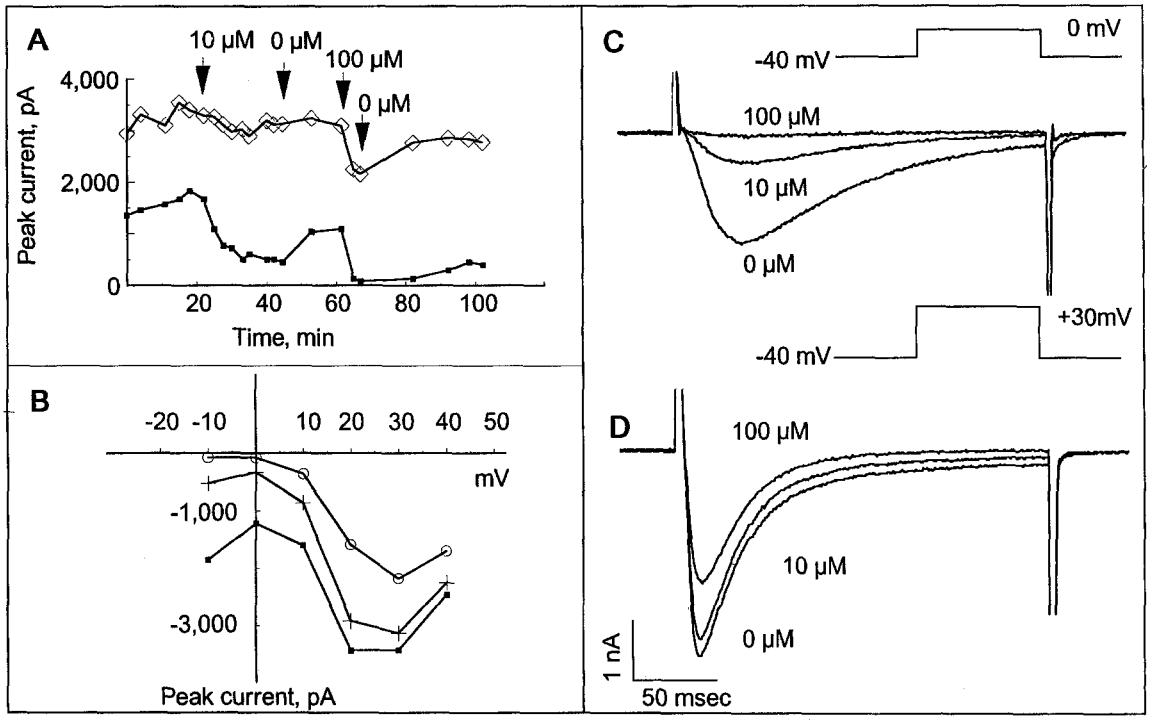

Fig. 6. Effect of nitrendipine on the two calcium currents. Both currents were recorded in 50 Ba-ASW from a holding potential of −40 mV. (*A*) Low threshold peak current at 0 mV (■) and high threshold peak current at +30 mV (◇) plotted *vs.* time during different applications of nitrendipine. (*B*) Corresponding current-voltage relations: no nitrendipine (■) 10 µM nitrendipine (+) and 100 µM nitrendipine (○). Representative traces of low threshold (*C*) and high threshold currents (*D*) recorded in three different concentrations of nitrendipine. Temperature 13°C.

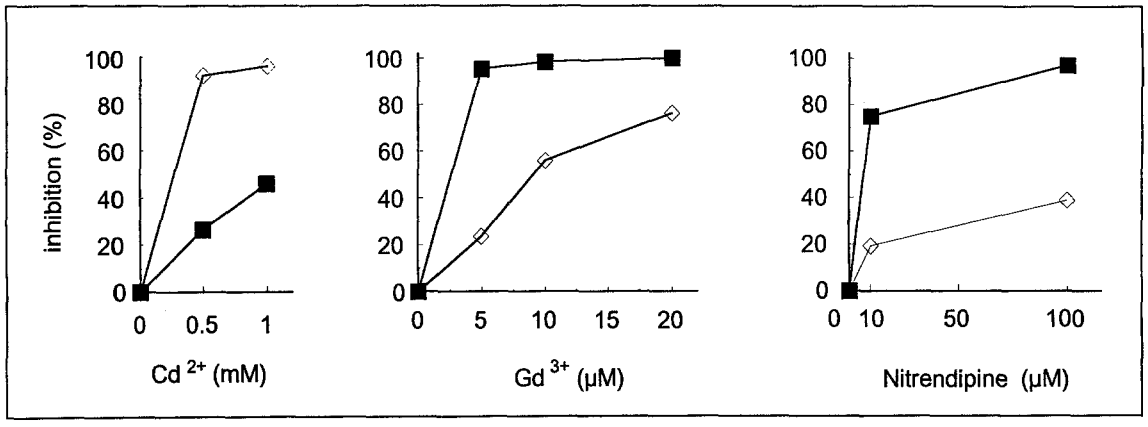

Fig. 7. Differential effects of cadmium, gadolinium and nitrendipine on the two calcium currents amplitudes. Records with same conditions that for Figs. 4, 5 and 6. (■) Low threshold current. (◇) High threshold current.

of the properties that are characteristic of the two major classes of identified calcium channels, the L-type and T-type. Both oocyte calcium currents are sensitive to a dihydropyridine, although the low threshold current is more sensitive and completely blockable while the high threshold is not. The lanthanide ion gadolinium preferentially blocks the low threshold current at concentrations similar to those required to block calcium currents in other cells. On the whole, if our purpose had been restricted to assign a single family to each of the two currents of the *Ciona* oocyte based on conventional criteria to distinguish between calcium channels, it

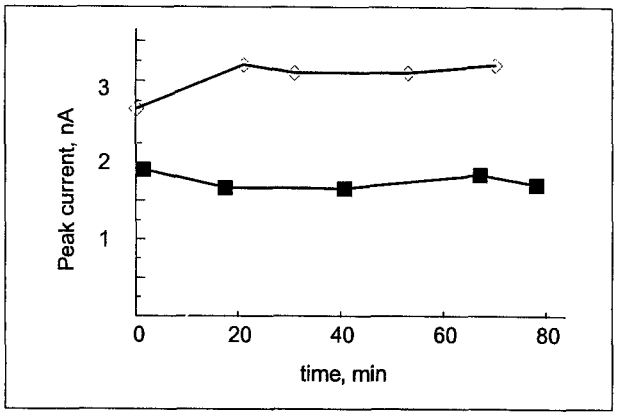

Fig. 8. Stability of the two calcium currents from one unfertilized egg. (■) Low threshold current. (◇) High threshold current. Holding potential −80 mV, temperature 15°C.

would not have been fruitful. It seems wise to adopt the position of Hille (1991): waiting for more calcium channels to be cloned to discuss subtypes based on structural data. In that respect, the similarity of behavior between the low threshold currents in different ascidians, like the sodium current of *Boltenia* and the calcium current of *Ciona,* suggests speculation for a high homology in their sequences and a natural mutagenesis, paralleling the laboratory-generated mutagenesis transforming a sodium channel into a calcium channel (Heinemann et al., 1992).

Our results did not depend on the geographical origin of animals, though some variations occurred between batches of animals from the same region. The two calcium currents that compose the total inward current stimulated by depolarization could be separated by carefully choosing holding potential, paradigm of voltage stimulation, and temperature. The *I-V* plots helped to quantify this separation when necessary. No contamination of the low threshold current was observed by the high threshold component. On the other hand, the low threshold current could contribute to a small percentage of the high threshold peak current. That would not affect the conclusion concerning the loss of the low threshold calcium current. We took advantage of the classical prescription of barium substitution for calcium, without the concerns mentioned by Dale et al. (1991). Some fertilized eggs never proceeded beyond furrow formation to a complete cleavage at the time of first cell division. This failure to divide might be due to a long-term effect of barium or to some disturbance caused by long-term patch-clamp recording.

The major finding of this study is the selective loss of the low threshold calcium current of *Ciona* oocyte after fertilization. The loss of a calcium channel could simply be a result of rundown, classically described in several cells (Fenwick, Marty & Neher, 1982; Chad &

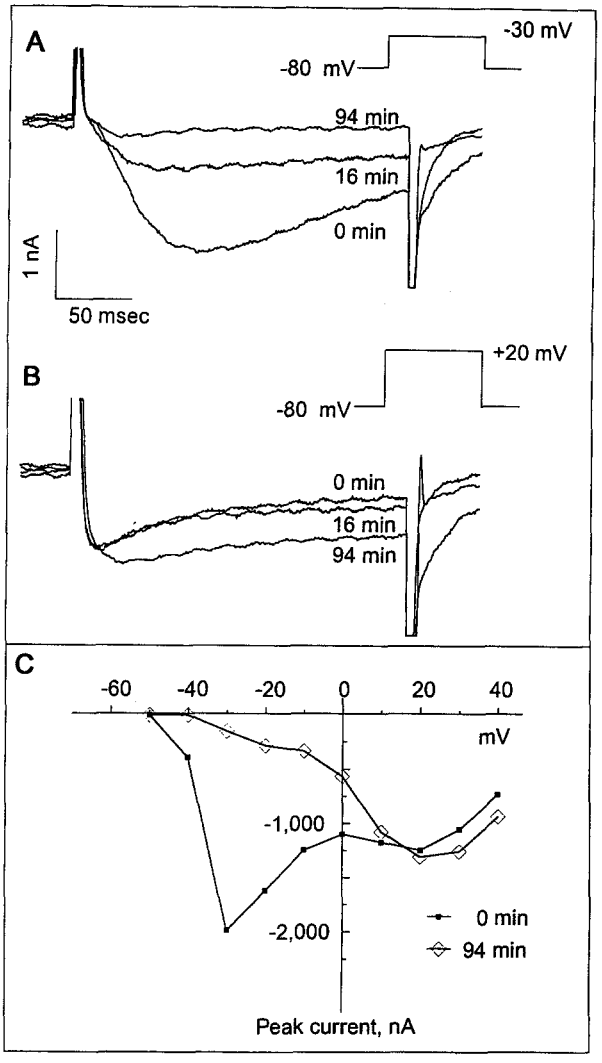

Fig. 9. The two calcium currents after fertilization. (*A*) Traces of low threshold current recorded at −30 mV just before (0 min), then 16 and 94 min after fertilization. (*B*) Traces of high threshold current at +20 mV at the same times. (*C*) Evolution of the current-voltage relation: just before fertilization (■) and 94 min after fertilization (◇).

Eckert, 1986). Our long-term recording experiments show that if any uncontrolled rundown was operating, it would have to be silent for hours before fertilization. Rejecting such an unlikely rundown, the loss of this low threshold calcium current is unequivocally triggered by fertilization. Our ability to prevent rundown and other disturbances to the oocyte is certainly due to the poor exchange between pipette and cell cytoplasm, given the small diameter of our pipettes in relation to these large cells (150 μ diameter). Finally, we also allowed oocytes to develop without recording until time of first cleavage and recorded no low threshold current.

The loss of low threshold excitability seems to be a common property at precise steps in development. In

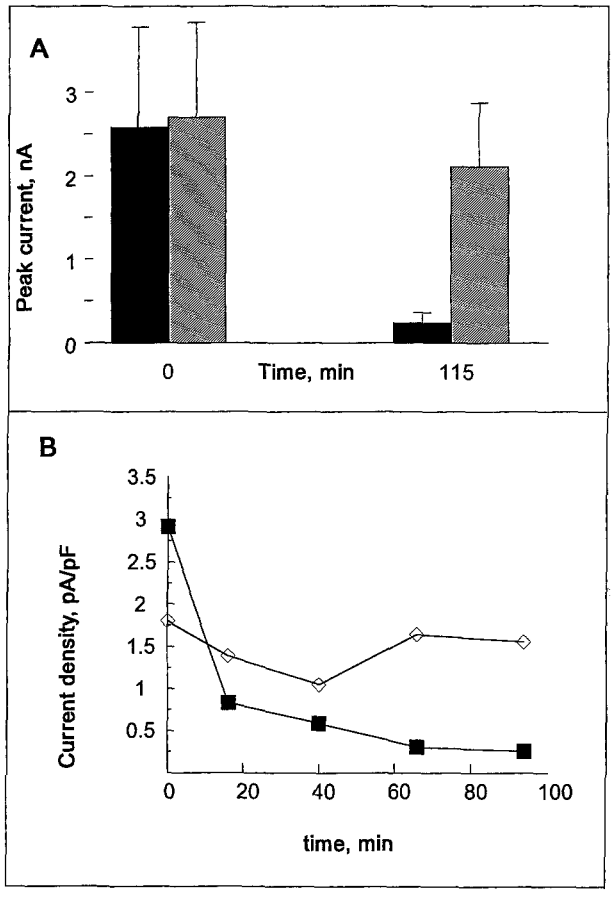

Fig. 10. Loss of low threshold current between meiosis resumption and first cleavage. (*A*) Histograms of peak current amplitudes (mean ± SD) of the two calcium currents before fertilization (n = 17) and after fertilization (n = 10) at time approaching first cleavage. Low threshold current (black) and high threshold current (hatched). (*B*) Time course of density of calcium currents after fertilization of one *Ciona* egg; in this plot, the time 0 is the time just before fertilization. (■) Low threshold current. (◇) High threshold current.

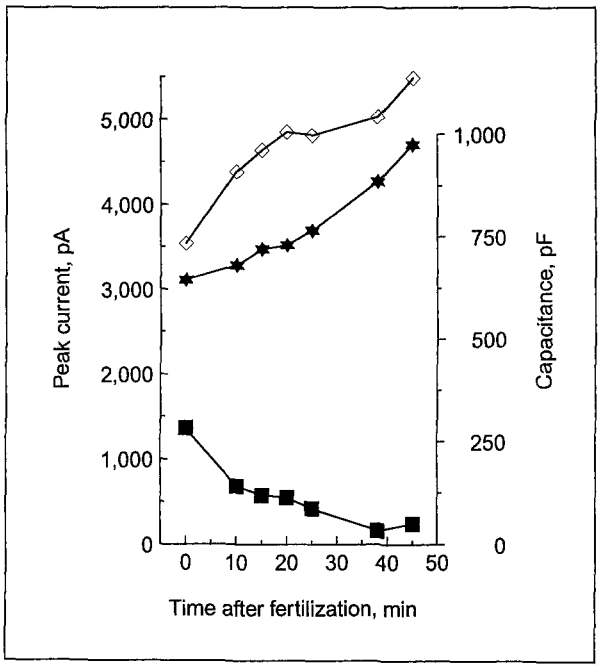

Fig. 11. Effect of emetine (100 μM) on calcium currents after fertilization. From a holding potential of −80 mV and at a temperature of 12°C, the two calcium currents were recorded respectively at −30 and +10 mV from the same fertilized egg maintained in an external solution of 50 Ba-ASW. (■) Low threshold current. (◇) High threshold current. (★) Capacitance.

early embryos, where inward currents may be cyclically activated at each cleavage, the loss of a low threshold current could prevent the egg from depolarizing up to threshold (Moody et al., 1991). The biological importance and the possible developmental significance of such a potential elimination of excitability is not established. At least, there is a striking parallel between our observation that fertilization triggers the loss of low threshold calcium current of *Ciona* and the observation that fertilization triggers the complete disappearance of a sodium current from *Boltenia* oocytes by the time of first cleavage (Block & Moody, 1987). The mechanism of this current loss remains unknown. In *Boltenia,* part of this reduction in amplitude has been shown, by experiments on merogones, to occur preferentially at the vegetal pole within the first tens of minutes after fertilization (Hice & Moody, 1988; Dale & Talevi, 1989). It does not account for the total amplitude loss, and several phenomena have to be invoked to explain the whole disappearance, despite the apparent monoexponential decay sometimes observed (Coombs et al., 1992). Internalization of membrane that contains channels might be a mechanism to suppress ion currents. We cannot rule out this explanation, although we recorded on *Ciona,* like on *Boltenia,* an increase of total membrane surface area while the low threshold currents declined. If there is a regulatory site on the channel to close it after fertilization, its action mechanism would not depend on any new synthesized protein since emetine did not prevent loss of calcium current, and neither would it depend upon a trypsin-like serine protease or a kinase, as TLCK and 6-DMAP did not affect the process. The loss of the low threshold calcium current, once fertilization is triggered, seems to be an unavoidable event that must be accomplished before further development occurs.

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