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Voltage-Dependent Inactivation of Calcium Currents in the Mouse Eggs

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= ABSTRACT =

Inactivation properties of Ca current in the unfertilized eggs of mouse were studied by using the whole cell voltage clamp technique and single microelectrode voltage clamp technique. Membrane potential was held at −80 mV and step depolarization was applied from −50 mV to 50 mV for 200~500 ms.

Peak of inward Ca currents was −2~−4 nA at a membrane potentials from −20 mV to 0 mV and outward currents were not observed within the membrane voltage range studied (−50~50 mV). Inward currents were fully inactivated within 200 ms after the onset of step depolarization. As the membrane became depolarized, time constant of inactivation (τ) was decreased but remained around 20~30 ms beyond 10 mV. When Ba^{2+} was used as a charge carrier, inactivation of inward Ba^{2+} current also occured and time course of inactivation was similar to that of Ca^{2+} currents as charge carrier. In the bathing solution containing high potassium (131 mM K^{+}), process of inactivation was not changed except a parallel decrease of value for the entire range of membrane potential. Steady-state inactivation of the current(h_{∞}) obtained from the double pulse experiment showed the voltage-dependent change. These results suggested that inactivation of Ca currents in the unfertilized eggs of mouse was voltage-dependent.

Key Word: Voltage-dependent inactivation, Whole cell voltage clamp, Mouse eggs.

INTRODUCTION

Inward currents observed in the unfertilized eggs were carried through the voltage-dependent Ca channels (Mitani, 1985; Okamoto et al, 1977; Peres, 1986; Peres, 1987). Relaxation after peak of the voltage-dependent Ca current was a common phenomena in the excitable cells. Such relaxation was also reported in the Ca currents of mouse eggs (Peres, 1987). There are two main possibilities to be considered about the mechanism of relaxation in the Ca current of mouse eggs. First, there could be an internal Ca^{2+}-dependent inactivation of Ca currents which have been studied in the *Paramecium* and *Aplysia* (Brehm & Eckert, 1978; Eckert and Tillotson, 1981; Yue et al, 1990). Second, there could be a voltage-dependent inactivation reported in the egg membrane of marine polychaetes, *Neanthes* (Fox, 1981; Sánchez and Stefani, 1983). Between the two possibilities, Ca^{2+}-induced type of inactivation was thought to be excluded because

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the time constant of inactivation (τ) was same for the both inward and outward currents and was independent of the current amplitude in mouse eggs (Peres, 1987). However, because there was no direct evidence that Ca currents were not Ca^{2+}-dependently inactivated, inactivation mechanism was still unclear in mouse egg.

To define the inactivation property, it is necessary to examine whether Ca conductance (gCa) was decreased or not in the course of current relaxation (Hille, 1984). If gCa is not altered, there would be no inactivation process (Almers et al, 1981). Also currents could decay by imposing an outward component on the inward currents. In the present work, we observed that the channel conductance decreased both with time and by the membrane potential and the relaxation of currents was not affected by the influx of Ca^{2+}. Thus this paper presents that inactivation of Ca currents is voltage-dependent in the unfertilized eggs of mouse.

METHODS

Mouse (Charles River strain of mixed breed, older than 4 weeks) was used as donor of the unfertilized eggs. Superovulation was induced by injection of 5 IU pregnant mare serum gonadotropin (PMSG, Sigma) followed by an injection of human chorionic gonadotropin (hCG, Sigma) via intraperitoneal route 60~65 hours and 12~17 hours, respectively before the collection of eggs. Eggs surrounded by the cumulus cells were collected from the ampullar part of oviduct. Cumulus mass and zona pellucida were removed by treatment with 0.1% hyaluronidase (Sigma, Type I-S) and by a exposure to 10~20 unit protease (Sigma) for 20 min in order to obtain a tight seal between the vitelline membrane of egg and pipette. However, zona pellucida of some eggs were not removed for the recording of currents by using the single electrode voltage clamp (switch clamp) technique. Zona-free eggs were washed out three times to avoid the protein digestion by protease.

Normal Tyrode solution used in the preparation of eggs contained following composition: 140 mM NaCl; 6 mM KCl; 1.2 mM $MgCl_2$; 2 mM $CaCl_2$; 20 mM HEPES; buffered to pH 7.3 with NaOH at 35°C. The bathing solution contained (in mM) 125 NaCl; 6 KCl; 2 $MgCl_2$; 20 $CaCl_2$; 20 HEPES-NaOH; pH 7.3. Pipette solution contained (in mM) 140 KCl; 2 $MgCl_2$; 1 $CaCl_2$; 10 Ethylene glycolbis (β-aminoethyl ether) N, N, N', N'-tetraacetic acid (EGTA); and buffered to 7.2 with KOH at 35°C (pCa 7~8). To prepare the high potassium (131 mM K^+) bathing solution, NaCl was replaced with the equimolar KCl. Concentration of $CaCl_2$ in the bathing solution was increased up to 20 mM to make the membrane stable and to facilitate the gigaseal formation (Peres, 1987).

Eggs were transferred into the 0.5 ml sized-chamber built in 35 mm petri dish and incubated for 5 minutes. Pipettes for the whole cell recording had a resistance of 2~3 MΩ in the bath. Resistance of microelectrodes 3M KCl) for the recording of currents using the switch calmp technique was 10~15 MΩ. After formation of gigaohm seal, condition of egg was reexamined on the stage of inverted microscope (CK-2, Olympus). Eggs which cytoplasmic contents was squeezed into pipette were excluded from the analysis. All eggs were used within 8 hours after collection from the oviduct and whole cell currents were recorded at room temperature.

Signals were digitized by both the analog-to-digital converter (Labmaster™ and TL-1-125, Axon) and a pulse code modulator (PCM-2, Medical Systems) and stored in a personal computer and video tape recorder, respectively. Current traces recorded by using the whole cell voltage calmp were leak-substracted while those by using the

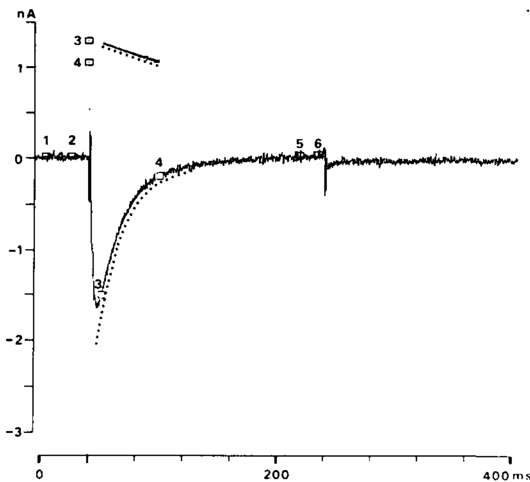

Fig. 1. An example of the exponential fitting in the course of current relaxation. Time constant of inactivation (τ) was calculated from the curve fitting program (pCLAMP, version 5.51, CLAMPAN, Axon). Regions between cursors represents the assignments in order to execute the exponential fit on this program. Region between cursor 1 and 2 represents to the baseline, between cursor 3 and 4 to fit and between cursor 5 and 6 to infinite-time. Two lines in the upper inset indicate the digitized data and the exponential function calculated, respectively on the semi-logarithmic scale. The vertical size between 3 and 4 in the upper box is corresponding to one decade.

switch clamp technique were not.

Leak components of the whole cell currents was subtracted by using the P/N protocol (pCLAMP, version 5.51, CLAMPEX, Axon). As shown in the Fig. 1, time constant of inactivation (τ) was obtained from the fitting the time course of the decays of Ca currents by a curve fitting program.

RESULTS

Calcium currents evoked in response to the step depolarizations were recorded and the current-voltage realtionship revealed that activation of currents was voltage-dependent in mouse eggs (Fig. 2). In this work, outward currents were hardly observed over the voltage range studied. The current traces reached peak within 5 ms after onset of the voltage step and inward currents after its peak rapidly decayed with time (Fig. 2 A). Such time-dependent decay seemed to be due to the decreased conductance of inward current for a net inward current rather than a increased conductance for a net outward currernt from the result shown in Fig. 3. When the egg membrane was clamped at −10 mV from the holding of −90 mV for 20, 25, 30, 40, 50, 60, and 80 ms before stepping to −80 mV by using the single electrode voltage clamp technique, the instantaneous tail currents were recorded and decreased with the duration of voltage pulse. The inward tail currents observed on repolarizing the egg to −80 mV near the holding potential were −4 nA when the depolarization was maintained for 20 ms. Tail current amplitude became smaller as the duration of voltage step was increased. But they were remained about −2.5 nA over 60 ms. Although the tail currents were contaminated by the capacitative currents of membrane due to the presence of zona pellucida, they reflected the instantaneous conductance when voltage step was interrupted (Fig. 3). Decrease of tail currents with the increase of step depolarization duration represented that there was changes in the conductance of inward currents.

Time constant of inactivation (τ) measured by using the fitting program (as shown in Fig. 1) was plotted against the membrane potential in Fig. 4. In panel A of Fig. 4, (τ) decreased from 201 ms at −30 mV to 42 ms at −10 mV and was kept about 25 ms beyond 10 mV. Similar voltage-dependency of τ was observed when the external Ca^{2+} was replaced by Ba^{2+}. When the external KCl was raised from the control solution (6 mM) to high potassium solution (131 mM), relationship between τ and membrane potential

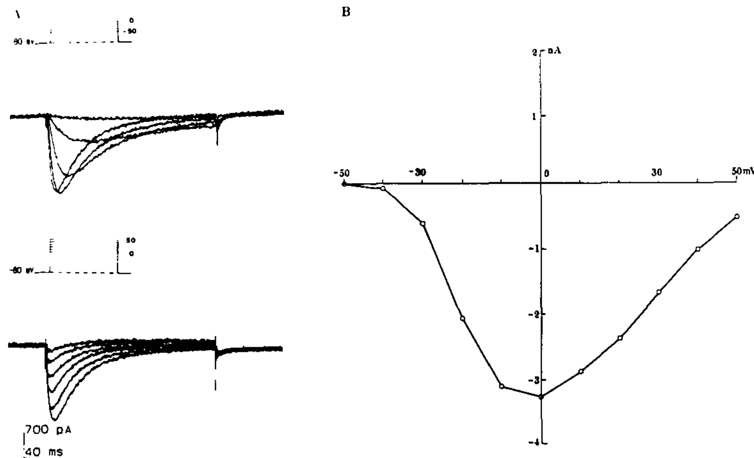

Fig. 2. Inward currents and current-voltage relationships in the unfertilized eggs of mouse. A, Traces of inward currents in response to step depolarization shown in the upper panel above the current traces. B, I-V curve. Currents traces in panel A and B were recorded in different eggs.

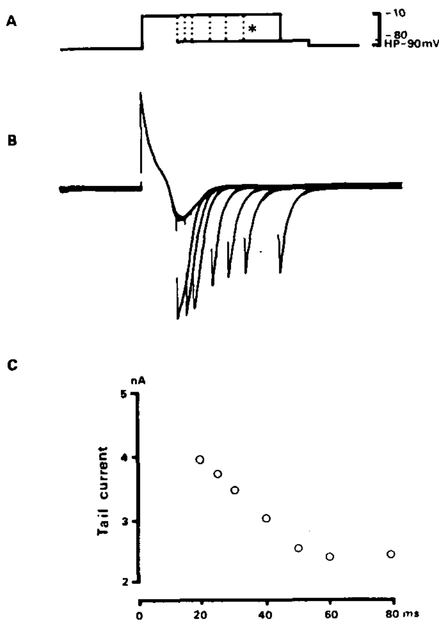

Fig. 3. Time-dependent tail currents evoked by step depolarizations with various duration (*). A and B, Protocol and traces observed. C, Relationship between the amplitude of tail currents and the duration of voltage step. These were recorded by using the single electrode voltage clamp technique in the zona-intact eggs. The tail currents were contaminated with the uncompensated capacitative current.

was not altered except the decrease of values about 5~10 ms (Fig. 4B).

Calcium conductance decreased in the course of current relaxation and values of τ were not changed by Ca^{2+} or Ba^{2+} as charge carrier. These facts suggested that inactivation of Ca currents did not depend at least on the internal Ca^{2+}. To confirm the voltage-dependent inactivation in Ca current of mouse eggs, we examined steady-state inactivation by using the double-pulse experiment shown in the panel A of Fig. 5.

Test pulse to 0 mV was preceded by 300 ms long conditioning prepulses given in every 20 mV step from −100 mV to 40 mV. Two pulse was seperated by a brief interval of 10 ms (protocol in upper panel of Fig. 5). Steady-state inactivation curve showed that the inward currents were almost inactivated at −20 mV (Fig. 5 C).

DISCUSSION

The present work was to observe the inactivation property of Ca currents in the mouse eggs. Because the inward currents

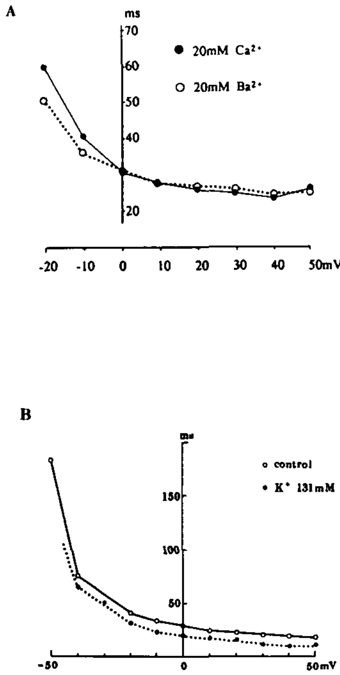

Fig. 4. Time constant of inactivation (τ) versus membrane potential. A, Comparison of the courses of τ in the bathing solution containing between Ca^{2+} and Ba^{2+}, respectively. B, Effect of varying $[K^+]_0$ on τ.

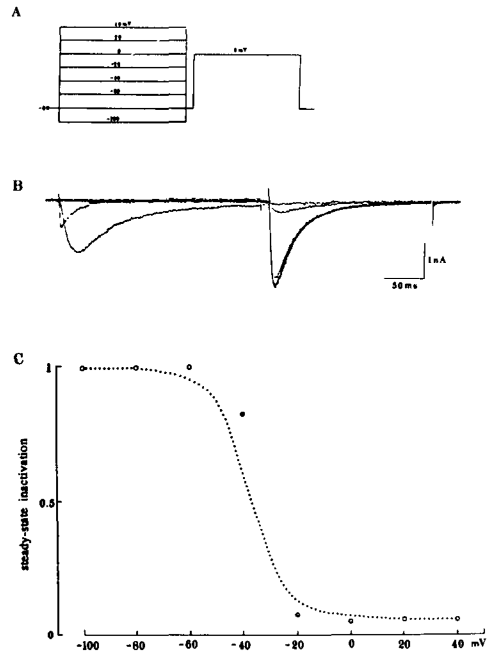

Fig. 5. Steady-state inactivation curve. A and B, Protocol of the double pulse experiment and the current traces recorded. C Steady-state inactivation curve obtained from the results in B. Dotted lines were drawn by eye.

observed in the mouse eggs were carried only by Ca^{2+} (Mitani, 1985; Okamoto et al., 1977; Peres, 1986), it is important to pay attention to the characteristics of Ca channels which play an essential role in fertilization. Also since it was already reported that the inactivation of inward currents through the Ca channels in mouse eggs was not at least Ca^{2+}-induced type of inactivation (Peres, 1987), this study was focused on observing the evidences about the voltage-dependent inactivation of Ca currents.

Several mechanisms underlying inactivation of Ca currents were proposed as follows (Brehm & Eckert, 1978; Eckert & Tillotson, 1981; Fox, 1981; Hille, 1984; Tsien, 1983); First, there could be no inactivation of the Ca conductance, rather, the relaxation process could be a turn on of a con-

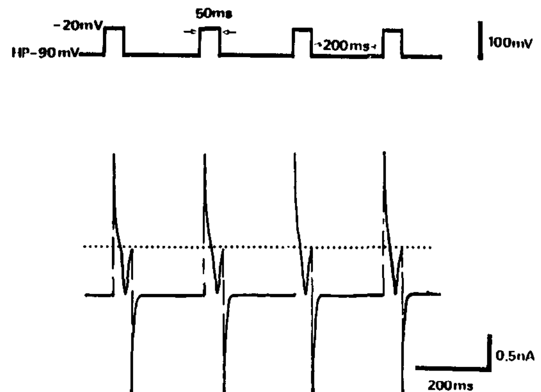

Fig. 6. Inward Ca currents elicited in response to the repetitive voltage step deploarizations. Each current was not altered in the amplitude and inactivation. Traces were recorded by use of single elctrode voltage clamp technique in a zona-intact eggs. Dotted line represents the zero-current level.

ductance that produces a net outward current and thereby masks the inward current and that makes it appear as if there is inactivation. Second, there could be a gCa decrease induced by the Ca current itself. Third, an external depletion or an internal accumulation of Ca^{2+} would decrease the gradient for Ca^{2+} across the membrane. Fourth, there could be a voltage-dependent inactivation of the gCa. From the results in this study, the assumption that there could be no inactivation of gCa could be excluded on the basis of the absence of outward currents over the voltage range (−50∼+50 mV, Fig. 2) and the presence of conductance decay (Fig. 3). Another possibility is later activation of outward component which contributes to the current relaxation during voltage step. If the activation of the outward conductance contributes to the relaxation of currents, the current relaxation should be slowed or disappeared when the concentration gradient for K^+ is reduced or removed. Under the equilibrated state for K^+ between inside and outside of the egg by the perfusion of high potassium media (131 mM KCl), time constant of inactivation (τ) was even decreased about 10 ms (Fig. 4B).

With the present results, however it could not be explained why the inactivation process was faster under the high K^+ media.

It is necessary to confirm that either Ca^{2+}-induced inactivation or voltage-dependent inactivation contributes to the relaxation of currents, or that both types be ascribed to the inactivation of currents in mouse eggs as shown in the ventricular myocytes of guinea pig (Lee et al, 1985). If the Ca conductance was decreased by the accumulation of intracellular Ca^{2+}, amplitudes of Ca current in response to the repetitive voltage step should be reduced or relaxation of those should be faster. Result in Fig. 6 suggested that accumulation of internal Ca^{2+} do not affected on the Ca conductance in the eggs based on the fact that currents were not altered in the amplitude and relaxation by the repetitive step depolarizations given in every 200 ms. Time interval, 200 ms is too short for intracellular Ca^{2+} to be removed outside cell or sequestrated into celluar organelles because one cycle of Ca^{2+} oscillation was continued at least for 20 sec in mouse eggs (Peres, 1990). Instead, steady-state inactivation curve was dependent on the membrane potential (Fig. 5). Therefore, observation in the present work indicated a voltage-dependent inactivation in the unfertilized eggs of mouse.

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