Kor J Physiol (1994) 28(2), 215-224

Calcium Current in the Unfertilized Egg of the Hamster

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=ABSTRACT=

The presence of a calcium current ($i_{Ca^{2+}}$) passed via a specific channel was examined in the unfertilized hamster egg using the whole-cell voltage clamp technique. Pure inward current was isolated using a Cs^+-rich pipette solution containing 10 mM TEA. This current was independent of external Na^+ and was highly sensitive to the Ca^{2+} concentration in the bathing solution, indicating that the inward current is carried by Ca^{2+}.

The maximal amplitude was -4.12 ± 0.58nA (n=12) with 10mM Ca^{2+} at -30mV from a holding potential of -80mV. This current reached its maximum within 20ms beyond -30mV and decayed rapidly with an inactivation time constant (τ) of 15ms. Activation and inactivation of this $i_{Ca^{2+}}$ was steeply dependent on the membrane potential. The $i_{Ca^{2+}}$ began to activate at the lower voltage of -55 mV and reached its peak at -35 mV, being completely inactivated at potentials more positive than -40 mV. These result suggest that $i_{Ca^{2+}}$ in hamster eggs passes through channels with electrical properties similar to low voltage-activated T-type channels. Other results from the present study support this suggestion; First, the inhibitory effect of Ni^{2+} (IC_{50}=13.7 μM) was more potent than Cd^{2+} (IC_{50}=123 μM). Second, Ba^{2+} conductance was equal to or below that of Ca^{2+}. Third, $i_{Ca^{2+}}$ in hamster eggs was relatively insensitive to nifedipine (IC_{50}=96.6 μM), known to be a specific L-type blocker.

The physiological role of $i_{Ca^{2+}}$ in the unfertilized hamster eggs remains unclear. Analysis from steady-state inactivation activation curves reveals that only a small amount of this current will pass in the voltage range ($-70\sim-30$ mV) which partially overlaps with the resting membrane potential. This current has the property that it can be easily activated by a weak depolarization, thus it may trigger a certain kind of a intracellular event following fertilization which may cause oscillations in the membrane potential.

Key Words: Calcium current, T-type channel, Unfertilized hamaster egg, Whole-cell voltage clamp technique

INTRODUCTION

Intracellular Ca^{2+} plays a critical role in muscle contraction, neurotransmitter release at synapses, cellular responses to several hormones, and in the cell division cycle (Alberts et al, 1989; Hille, 1992; Reuter, 1983). Changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) level have been reported in differentiation of various cells (Arnoult & Villaz, 1994; Ciapa et al, 1994; Miyazaki et al, 1986; Shiina et al, 1993). However the importance of these changes remains

unclear, even in the fertilization process of mammalian eggs. Previous studies suggested that the initial increase in $[Ca^{2+}]_i$ was closely related to the synthesis of cortical granules which release a substance to produce polyspermy block (Alberts et al, 1989). In hamster oocytes, characteristic hyperpolarization responses (HRs) were frequently observed during sperm-binding with an egg *in vitro* (Miyazaki & Igusa, 1981; Miyazaki & Igusa, 1982). This HR reaction occurred following a rise of Ca^{2+} inside the mammalian egg (Igusa & Miyzaki, 1986; Miyazaki et al, 1986; Miyazaki, 1991). It is thought that this reaction was due to the elevation of Ca^{2+}-dependent K^+ conductance (Miyazaki & Igusa, 1982; Yoshida et al, 1990).

The inositol triphosphate ($InsP_3$)-sensitive Ca^{2+} pool has been generally accepted as the main source of the increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) at fertilization (Miyazaki, 1988; Miyazaki et al, 1992a; Miyazaki et al, 1992b). Sperm binding with an egg cannot lead to the fertilization process and further division in a Ca^{2+}-free condition. Therefore external Ca^{2+} is an essential factor for fertilization in physiological conditions. Ca^{2+} removal from the experimental media results in a loss or a decrease of HR in eggs (Igusa & Miyazaki, 1983). A Ca^{2+} spike could be elicited and this was followed by anode-break excitation (Georgiou et al, 1984: Hong et al, 1991). These results suggest that there is an pathway for Ca^{2+} entry through the membrane of the egg and that this entry may be regulated by the membrane voltage. If external Ca^{2+} entered via Ca^{2+} channels, it would be possible to change $[Ca^{2+}]_i$ Via Ca^{2+} entry through Ca^{2+} channels as well as $InsP_3$-sensitive pool inside eggs at fertilization. Few report has been made about the presence of Ca^{2+} channels and their characteristics.

Ca^{2+} channels can be divided into several types, such as L-, N-, T- and P-type (and also others such as Q- and R-type) according to the classification criteria (Kostyuk, 1989; Llinas et al, 1992; Tsien et al, 1988; Tsien & Tsien, 1990). These criteria do not provide direct information about the function of each of the channel types. The physiological role of a channel type may be deduced from their distribution and the cell types which contain a certain type of Ca^{2+} channel, (Tsien et al, 1988). The present study was performed to demonstrate the presence of a Ca^{2+} current in hamster eggs and to clarify the type of channel carrying this current according to the pharmacological and biophysical criteria for T-, L- and N-type, using the whole cell voltage clamp technique.

Here we, for the first time, report the presence of a Ca^{2+} current in the hamster egg through channel which has properties similar to those seen in T-type channels.

METHODS

Chinese golden hamsters, older than 6 weeks, were used as egg donors. Hamsters were treated to induce ovulation with an intraperitoneal injection of 20 units of pregnant mare serum gonadotropin (PMSG) and of human chorionic gonadotropin (HCG). Eggs were collected from the oviduct 12~17 hours later following the HCG injection. The cumulus cell and zona pellucida surrounding the eggs were removed by applying hyaluronidase (100 units, Sigma) and one unit of protease (type VIII, Sigma), respectively at 24~26°C. Subsequently, the zona-free egg was washed several times and incubated with a medium containing (in mM): NaCl, 140; Na-pyruvate, 0.1; Lactate, 10; (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, HEPES), 20; $MgCl_2$, 1.2; $CaCl_2$, 2.0; KCl, 6.0; and 2 mg/ml polyvinylpyrrolidone. These were then transferred to 35 mm plastic Petri dishes. They were kept in a refrigerator (4°C) before use. In this experiment, current recordings were performed on eggs less than 8

hours after collection from the oviduct.

The standard bathing solution contained (in mM): NaCl 130; KCl, 6, Na-pyruvate, 0.1; Lactate, 10; $MgCl_2$, 1.2; $CaCl_2$, 10; HEPES, 20 (pH 7.4 at 37°C). In some experiments, equimolar $BaCl_2$ replaced $CaCl_2$. The pipette solution contained (in mM): CsCl, 120; tetraethylammonium chloride (TEA-Cl), 20; $MgCl_2$, 1.0; HEPES, 10; ditris creatine phosphate, 5.0; Mg-ATP, 1.0 (pH 7.4 at 37°C). All chemicals were purchased from Sigma (USA).

An unfertilized egg from the Petri dish was transferred to the experimental chamber housed in the inverted microscope (CK-2, Olympus, Japan) and it was then left in the bathing solution at least 5 min before the patch study began. The superfusion rate of the bathing solution was kept steady at less than 1 ml/min. The resistance of the patch pipette tip was 2~3 MΩ. After establishment of giga-seal, the patch membrane was usually disrupted by additional negative pressure. On forming the whole-cell patch configuration, the condition of the egg was examined visually: eggs in which the cytoplasm was squeezed into the pipette interior were discarded.

Currents were recorded using whole-cell voltage clamp technique. The membrane voltage of the egg was held at -80 mV. A series of step depolarizations going to $+50$ mV from -60 mV were given in 10 mV steps. Each step depolarization was 250 ms long and was applied every 15 sec. To obtain the steady-state inactivation curve, a double-pulse protocol was used; the membrane of the egg was clamped at -100 mV and a 200 ms-long test pulse to -30 mV was applied following by the prepulse of 10 mV (250 ms). All experiments were carried out at 24~26°C.

Whole cell currents were recorded via a patch clamp amplifier (CEZ-2100, Nihon Koden, Japan) and were monitored by a digital oscilloscope (CS-8010, Kenwood, Japan) and were digitized by an analog-to-digital converter (Axolab 1100, Axon, USA) and were then stored on a personal computer. Stimulation, data aquisition and analyses were performed with a software program (pClamp version 5.51, Axon, USA).

The average size the eggs after enzymatic digestion was approximately 50 μm and the capacitance was 700~800 pF with a standard deviation of less than 12% (n=16) in a 2 mM Ca^{2+} bath solution. Also, the standard deviation of the current was normalized to give a capacitance less than 15%. Hence, the current amplitude in this study was recorded without normalization of capacitance. Currents obtained in the egg with a standard deviation more than range above mentioned above were eliminated. Data are represented as mean±standard deviation (SD) with the number of observations.

RESULTS

In a preliminary patch study, both inward and outward currents were shown in the unfertilized egg of the hamster. Since there have been few reports about these currents, we hypothesized that either Ca^{2+} or Na^+ might carry the inward current, (and K^+ for the outward currents), based on the general observation of a wide range of cells within a physiological environment. To block the K^+ component of the currents, the pipette solution contained Cs^+ instead of K^+ and 20 mM TEA. This study is the first examination of the electrophysiological properties of hamster eggs. We therefore applied voltage pulse protocols which were used in a similar experiment performed on mouse eggs (Peres, 1987; Yang et al, 1993).

Representative current traces and a current-voltage (I-V) relation are shown in Fig. 1. In a condition that blocked the K^+ channels, it was found that only inward currents were elicited in response to step depolarizations (Fig. 1A). This

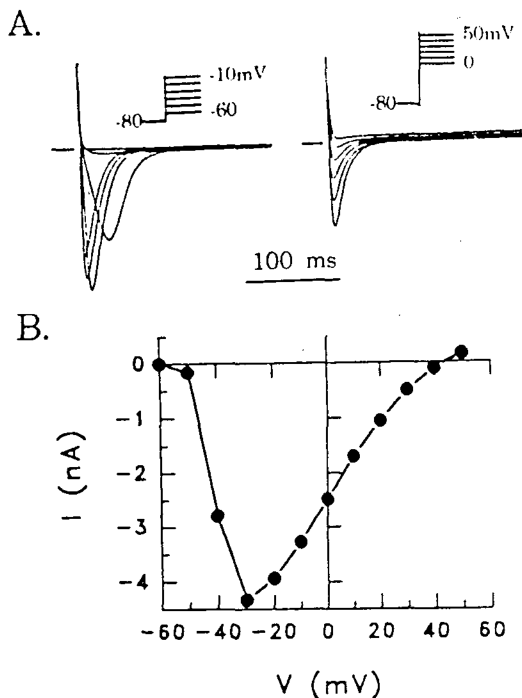

Fig. 1. Representative current traces in hamster eggs. A, traces of inward currents elicited by voltage pulses from -60 *mV to* -10 *mV (left panel) and from 0 mV to 50 mV (right panel) for 300 ms. B, The current-voltage (I-V) relation for inward currents. Membrane was kept at* -80 *mV.*

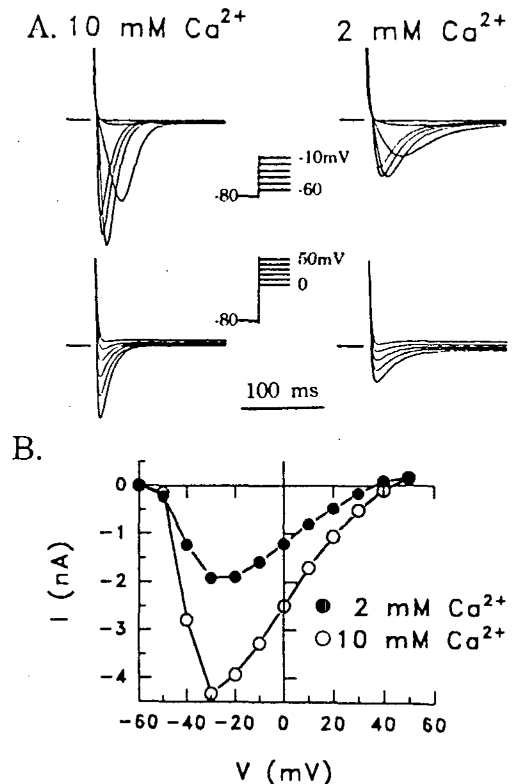

Fig. 2. Effect of external Ca^{2+} *concentration on the inward currents. A, representative traces responding to 10 mM* Ca^{2+} *(left) and 2 mM* Ca^{2+} *(right). B, The I-V relations from two series of traces recorded by application of same pulse protocol (center in A). Parameters in this I-V relation did not change except the current amplitude at corresponding potentials. This was recorded in the same egg.*

inward current was elicited by a voltage pulse more positive than -60 mV, and reached its maximum within 20 ms and then subsequently inactivated to a zero current level (Fig. 1A). This time course was much slower than those of well-known Na^+ currents recorded in skeletal muscle or other excitable cells. The current-voltage relation showed that this current appeared more positive than -60 mV and reached its maximum at -30 mV. It reversed around 45 mV.

Inward currents are generally carried by either Na^+ or Ca^{2+}. To discriminate which ion carried the inward current shown in Fig. 1, changes in current amplitude or its shape were investigated in Na^+-free medium, where 130 mM Na^+ was replaced by equimolar choline, which is membrane impermeant. The parameters of the current were not significantly changed, suggesting that Na^+ may not be the main component carrying this inward current (data not shown). However this current was sensitive to the external Ca^{2+} concentration ($[Ca^{2+}]_o$). When $[Ca^{2+}]_o$ was reduced from 10 mM to 2 mM, the maximal amplitude at -30 mV was decreased from 4.12 ± 0.58 nA (mean $\pm$ S.D., n=12) to 1.70 ± 0.30 nA (n=3) as shown in Fig. 2 A. However these changes in $[Ca^{2+}]_o$

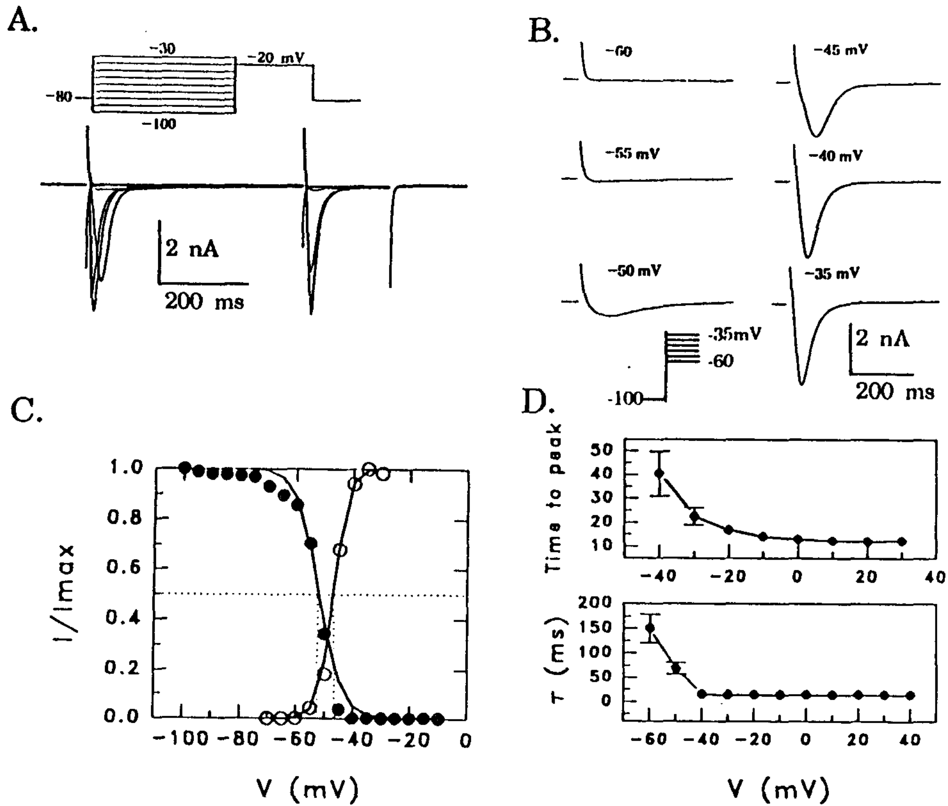

Fig. 3. Steady-state inactivation and activation curves. A, currents evoked by the double-pulse protocol in upper panel. B, inward currents by step depolarization with 5 mV intervals from −60 mV to −35 mV. C, steady-state inactivation and activation curves. Experimental data from A, normalized to current maximum obtained by preceding −100 mV prepulse (●) is plotted against the voltage of prepulse (V) for steady-state inactivation curve. For activation curve, Data from B, normalized to current maximum at −35 mV step pulse (O) is plotted against the step depolarization represented upon the each current trace. Data were fitted with Boltzman equation, $I/I_{max} = 1/[1+\{(V-V_{1/2})/k\}]$ *for inactivation curve and* $1/[1+\{-(V-V_{1/2})/k\}]$ *for activation curve. D, Plots of time to peak (upper panel) and inactivation time constant (lower panel) against membrane potential studied. Unit is ms for both curves.*

did not change any parameter except amplitudes in I-V relation (Fig. 2 B). These results indicate that inward currents in the hamster egg may be carried via channels specific for Ca^{2+}.

To examine further the voltge-dependent properties of this inward current, both steady-state inactivation and activation curves were obtained (Fig. 3). After a prepulse to −100 mV, the inward current reached its maximum in response to the test pulse to −20 mV (upper panel in Fig. 3 A). The more depolarized the prepulse, the smaller the amplitude of the current. The current amplitudes (I) elicited by test pulses to −20 mV (200 ms) following various 250 ms-long prepulses were normalized to that obtained with the preceding pulse to −100 mV (I_{max}) and plotted against the voltage of the prepulse in Fig. 3 C as closed circles. These data were fitted with Boltzmann equation,

$$I/I_{max} = [1+\exp\{(V-V_{1/2})/k\}]^{-1}$$

where V represents the voltage of the prepulse; $V_{1/2}$, the voltage of half inactivation; and k, the slope. The data were well fitted by this equation where $V_{1/2}$ and k were −53 mV and 3.5 mV, respectively. Around the −40 mV, the channels were completely inactivated, as seen on this curve (Fig. 3C). The steady-state activation curve was plotted using the current amplitude normalized to the maximum elicited by a step depolarization to −35 mV from a holding potential of −100 mV (upper inset in Fig 3 B). These data were also fitted by the Boltzmann equation,

$$I/I_{max} = [1 + \exp\{(V - V_{1/2})/k\}]^{-1}$$

where $V_{1/2}$ was −47 mV and k was 2.5 mV (open circles in Fig. 3 C). From −60 mV to −35 mV where the channels are still activated but not fully inactivated, a small amount of $i_{Ca^{2+}}$ can flow (i.e. as window current) into the egg.

The most striking feature of this inward current was its transient activation and inactivation, in contrast to long-lasting L-type currents. The current reached its maximum within 20 ms beyond −20 mV. The current relaxation curve was well fitted by a first order of exponential function. The inactivation time constant was less than 15 ms in the voltage range tested (−60 mV～+40 mV, Fig. 3D).

Ca^{2+} channels can be generally classified according to their kinetics and/or pharmacological criteria. The physiological role of each type in Ca^{2+} channels can be deduced from the function of the cell in which a type of Ca^{2+} channel is found. To date, the physiological role of Ca^{2+} current ($i_{Ca^{2+}}$) in the hamster egg remains unclear. We examined the electrical and the pharmacolgical properties of $i_{Ca^{2+}}$ in the unfertilized hamster egg to investigate its function further using the criteria reported by Tsien' s group (Fox et al, 1987; Tsien et al, 1988).

In Fig. 1 and Fig. 2, inward current was not only activated transiently but also began to be activated at the lower voltage of −50 mV with a holding potential of −80 mV. When the membrane was held at a potential positive to -50 mV, these inward currents did not appear even with strong depolarizations (see Fig. 3 C). This indicates that the Ca^{2+} current in hamster eggs might be a low-voltage activated type, i. e. close to the electrical characteristics of T-type. Thus we tested the Ba^{2+} permeability, because most Ca^{2+} channels except T-type are more highly permeable to Ba^{2+} than to Ca^{2+} (Tsien et al, 1988). Equimolar Ba^{2+} (10 mM) instead of Ca^{2+} did not increase the amplitude of the inward current (−1.99 nA in 2 mM Ca^{2+} versus −1.94

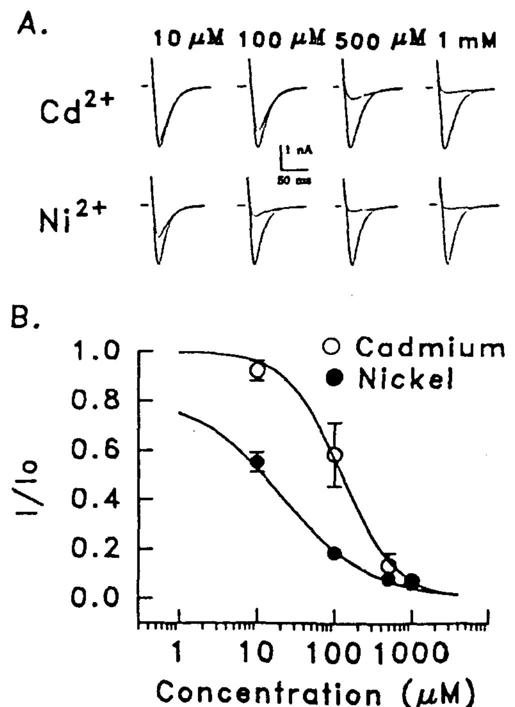

Fig. 4. Comparison of the blocking potency between Ni^{2+} and Cd^{2+} on the inward current. A, superimposed traces elicited by voltage pulse to −30 mV from holding potential of −80 mV before and after adding divalent blockers with concentration indicated on current traces, Cd^{2+} in upper and Ni^{2+} in lower panel. B, dose-response curves between divalent blocker and currents normalized to those (I_o) in the absence of the blocker. Data were fitted by the equation, $I/I_o = 1/\{1 + ([X]/[X_{1/2}])^n\}$, where X is the concentration of blocker, $X_{1/2}$ represents IC_{50} and n is for the slope of curve.

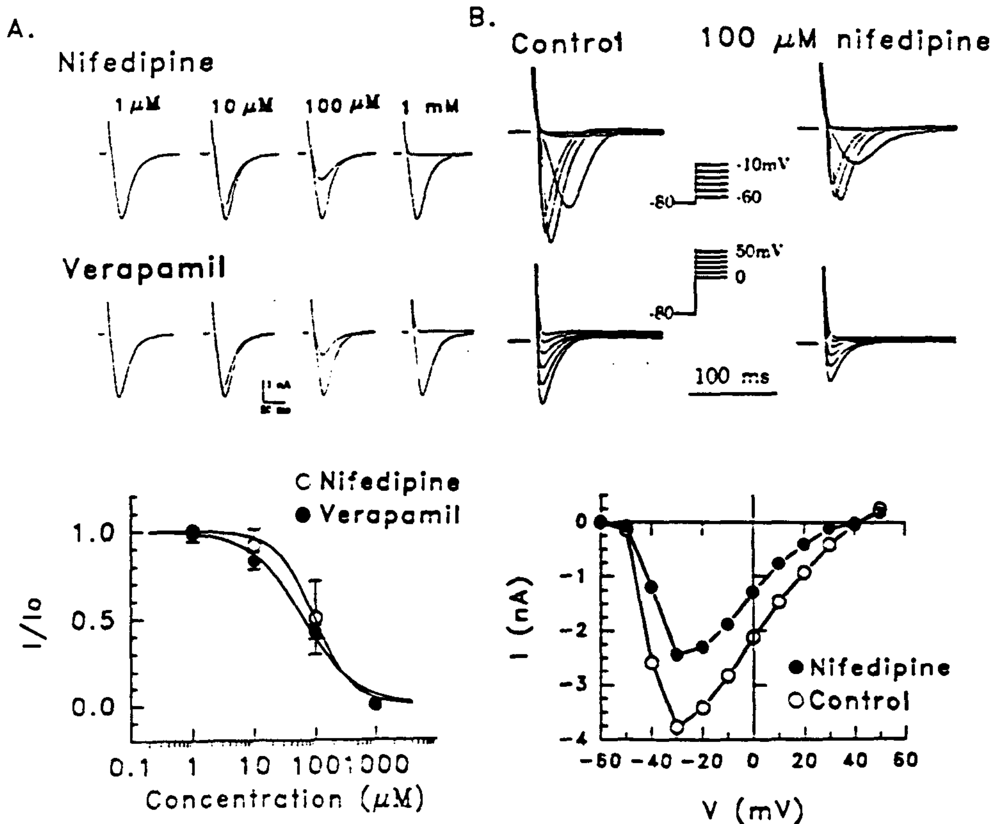

Fig. 5. Effect of organic blockers on the inward current. A, current traces in the presence and absence of nifedipine and verapamil in upper panel. Fractions of decrease in amplitude by nifedipine or verapamil are plotted versus the concentration of blockers. Data were fitted by the same equation shown in Fig. 4. B, inward currents resistant to 100 μM nifedipine. Currents elicited by voltage pulses are shown in left upper (control) and right upper panel (in the presence of 100 μM nifedipine). I-V relations of inward currents measured with and without nifedipine.

nA in 2 mM Ba^{2+} at −10 mV) and did not change the I-V relation or the parameters of the current. Another index which discriminates between high-threshold types and low-threshold type is the sensitivity to inorganic blockers, such as Ni^{2+} and Cd^{2+}. Ni^{2+} blocks the low-threshold type more potently than Cd^{2+} does. Figure 4 showed that the inward current was reduced to half of its maximum by 10 μM Ni^{2+} in the bath (IC_{50}: 13.7 μM) but it was decreased to 50% in the presence of 100 μM Cd^{2+} (IC_{50}: 123 μM), suggesting that this current was more sensitive to Ni^{2+} than to Cd^{2+}.

Dihydropyridines (DHPs) are known as specific high-threshold Ca^{2+} channel blockers, although this type of channel has different sensitivities to DHPs in different cells. We tested the effect of nifedipine. Nifedipine blocked the inward current with an IC_{50} of 96.6 μM. The inhibitory effect of verapamil was similar to that of nifedipine (IC_{50}: 63 μM) as shown in Fig. 5. A remarkable reduction in the inward current amplitude was observed with 100 μM nifedipine over the entire range of voltage pulses (from −40 mV to 40 mV). However there were no changes in the I-V relation except for the current

amplitude (Fig. 5B). In the double pulse experiment, the recovery time of the inward current was measured. The amplitude of the current elicited by a second pulse (step depolarization of -30 mV from holding potential of -80 mV) was fully recovered 1300 ms following the first pulse in the absence of nifedipine, while it had not recoverd even 4 sec after applying the first pulse in the presence of 100 μM nifedipine.

DISCUSSION

The present study shows the existence of a Ca^{2+} current in the unfertilized egg via channels with characteristics close to T-type channels. This can be supported by several evidences; Firstly, inward current was independent of $[Na^+]_o$, steeply sensitive to changes in external bath Ca^{2+} concentration (Fig. 2). Secondly, this current was activated at relatively negative potentials and inactivated rapidly in a voltage-dependent manner (Fig. 3). Thirdly, the conductance for Ba^{2+} was equal or below that for Ca^{2+} and the inhibitory effect of Ni^{2+} on this current was more potent than that of Cd^{2+} (Fig. 4). Finally, this current was relatively insensitive to nifedipine, a representive DHP antagonist (IC_{50} was about 96.6 μM). Whilst a submicromolar level of nifedipine is enough to inhibit L-type Ca^{2+} current in some cells (Fox et al, 1987; Nowycky et al, 1985), nearly 100 μM nifedipine is needed to reduce Ca^{2+} current ($i_{Ca^{2+}}$) by a half in hamster eggs (Fig. 5). Since the concentration of a DHP antagonist is not decisive factor to determine channel types, however we also tested the effect of Bay K 8644 on this inward current in a preliminary study. If L-type Ca^{2+} channels exist on the egg membrane, the current should have increased in the presence of Bay K 8644 (Lemos & Nowycky, 1989). Inward current did not respond to Bay K 8644. We did not try to classify this current into P-, Q- and R-type in detail, however.

The existence of $i_{Ca^{2+}}$ in the unfertilized mammalian egg gives the possibility that this current may contribute to the egg homeostasis and fertilization. Unfertilized eggs in the oviduct go through degeneration tens of hours after ovulation without coupling to sperm (Hafez, 1980). In fact, the resting membrane potential (RMP) of the egg is generally in $-20 \sim -40$ mV (Igusa & Miyazaki, 1983; Miyazaki & gusa, 1982), a voltage range where Ca^{2+} channels are almost inactivated, meaning that Ca^{2+} cannot flow into the egg interior (see Fig. 3). Thus, it is possible that the activation of this channel could play a role in fertilization. This assumption can be supported by the fact that $i_{Ca^{2+}}$ decreases following fertilization (unpublished data). Supposing the general function of the Ca^{2+} channel were to serve as one of pathways for Ca^{2+} influx, then this channel should be activated by hyperpolarization occurring early on in fertilization, meaning external Ca^{2+} could flow into the egg. Previously there has been no evidence that Ca^{2+} moves through voltage-gated channels in the mammalian egg. Only Ca^{2+} release from internal store via the stimulation of inositol 1,4, 5-triphosphate ($InsP_3$) has been proven as a route to raise the intracellular Ca^{2+} level (Miyazaki et al, 1992a; Nuccitelli et al, 1993). However, as we can see from the curves in Fig. 6 C, a small amount of (window) current can pass through the channel if the off-response following hyperpolarization (ca. 10 mV below RMP) might occur during coupling to sperm. This current could then contribute to the $[Ca^{2+}]_i$ rise in addition to the $InsP_3$-stimulated Ca^{2+} increase.

In view of the physiological role generally accorded to the channel types, T-type channels are generally invoked as generators of pacemaker activity. Oocytes can neither be fertilized nor developed in a Ca^{2+}-free medium, suggesting that Ca^{2+} is the essential factor in embryonic differentiation. Thus it seems that the

Ca^{2+} channel in hamster eggs is more related to initiating the cascade leading to cell development. Therefore, activation of $i_{Ca^{2+}}$ in the unfertilized egg may be followed by coupling with sperm and may contribute to cell signalling in early differentiation rather than acting as a source of Ca^{2+} for the egg.

There are some differences in the electrical properties of hamster eggs compared to those of mouse eggs. Both eggs have $i_{Ca^{2+}}$ via channels close to T-type, the amplitude of $i_{Ca^{2+}}$ in hamster eggs is larger (ca −4.1 nA at 10 mM Ca^{2+}) than that in mouse eggs (−2.2 nA at 20 mM Ca^{2+}) (Yang et al, 1993). Considering the similar size both kinds of eggs of around 50 μm, it is possible that either channel density or channel coductance may be greater in the hamster egg than in the mouse egg. Although $i_{Ca^{2+}}$ in both kinds of eggs shows voltage-dependent inactivation, iCa^{2+} in the hamster egg inactivated faster than in the mouse egg. The inactivation time constants of hamster eggs and mouse eggs were 28 ms and 15 ms, respectively (Yang et al, 1993). If the kinetics of the channels in mouse and hamster eggs were similar, outward current (s) might occur later and mix with the inward current during its inactivation. Since this experiment was done under K^+ channel blockade, it could be excluded that the outward K^+ component might accelerate the decay of the inward current. It seems that in hamster eggs, Ca^{2+} channels have different kinetics or possibly other ions such as Cl- may be involved in the inactivation process.

The electrical characteristics of $i_{Ca^{2+}}$ in hamster eggs may be altered or lost during development because the unfertilized egg is an immature cell which becomes a fetus with various highly-differentiated channels via the embryonic stage. It is not possible to know the exact time of loss of properties of $i_{Ca^{2+}}$, or when new kinds of channels are expressed. At present, it seems possible that this type of $i_{Ca^{2+}}$ might exist up to the 1-cell stage after fertilization. In the mouse egg, $i_{Ca^{2+}}$ with an I-V relation similar to that of $i_{Ca^{2+}}$ in the unfertilized egg was reported (Hong et al, 1992), suggesting that $i_{Ca^{2+}}$ in hamster eggs may exist at least till 1-cell stage if they have a comparable progress in embryonic development.

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