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Low-Voltage Activated Ca^{2+} Current Carried via T-type Channels in the Mouse Egg

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= ABSTRACT=

Most of voltage-operated Ca^{2+} channels can be divided into three types (T-, N-, and L-type), according to the electrical and pharmacological properties. Their distribution is closely related to cell-specific functions. Properties of the voltage-activated Ca^{2+} current in mouse eggs were examined to classify channel types and to deduce the function by using whole-cell voltage-clamp technique.

Ca^{2+} currents appeared below -40 mV and reached a maximum at -15 mV (half maximum was -31 mV), then decayed rapidly (inactivation time constant, $\tau = 28.2 \pm 9.59$ ms at -10 mV) within 50 ms after the onset of step depolarization. Activation and inactivation of the Ca^{2+} channel was steeply dependent on voltage, in a relatively low range of -70 mV~-10 mV, half maximum of activation was -31 mV and that of inactivation was -39 mV, respectively. This current was not decreased significantly by nifedipine, a specific dihydropyridine Ca^{2+} channel blocker in the range of 1 μM to 100 μM. The inhibitory effect of Ni^{2+} on Ca^{2+} current was greater than that of Cd^{2+}. The conductance of Ba^{2+} through the channel was equal to or lower than that of Ca^{2+}. These results implied that Ca^{2+} current activated at a lower voltage in the mouse egg is carried via a Ca^{2+} channel with similar properties that of the T-type channel.

Key Words: Mouse egg, Ca^{2+}current, Ca^{2+}channel, T-type, Whole-cell voltage-clamp

INTRODUCTION

Ca^{2+} entry through Ca^{2+} channels can be facilitated by many kinds of stimuli, such as membrane depolarization, receptor-liganding, or physical changes of cell volume and pressure (Tsien & Tsien, 1990). Voltage-operated Ca^{2+} channels (VOCs) can be classified into several types according to their gating kinetics, ionic conductance and pharmacological properties (Tsien et al, 1988; Tsien & Tsien, 1990). Multiple types of VOCs have been found in many cells, both excitable and non-excitable. Their distribution is also complicated with a variety of cells (Kostyuk, 1989; Tsien et al, 1988). These findings led to the assumption that cell-specific function was closely related to the distributed channel types. Therefore, the physiological functions of VOCs may be deduced through the investigation of channel properties distributed in cells examined.

There have been few reports on the presence of i_{Ca} in the mouse egg (Okamoto et al, 1977; Peres, 1987; Park et al., 1991). However, properties of this current have not been well documented. This may be due to the difficulties in preparing the materials suitable to the electrophysiological recordings.

Ca^{2+} currents recorded in both fertilized and

unfertilized eggs of the mouse have been thought to be carried through channels highly selective to Ca^{2+} (Okamoto et al, 1977; Peres, 1986; Peres, 1987). They are activated in the lower voltage range and then inactivated rapidly (Peres, 1987; Park et al, 1991). Here we examined the properties of i_{Ca} and subsequently classified Ca^{2+} channels carrying i_{Ca} in mouse eggs according to the three (or four) major channel type (T-, N-, and L-type or T-, N-, L-, and P-type) (Nowycky et al, 1985; Fox et al, 1987; Tsien et al, 1988; Tsien & Tsien, 1990; Llinás et al, 1992;), and to deduce the role of these Ca^{2+} channels in fertilization.

In the present study, transient i_{Ca} in the mouse egg was activated at a relatively low-voltage, it was resistant to decrease by nifedipine, a dihydropyridine (DHP) Ca^{2+} channel blocker. Ni^{2+} was a more potent inhibitor of this current than was Cd^{2+} and the conductance in Ba^{2+} was simillar to that in Ca^{2+}. These results indicate that i_{Ca} in mouse eggs is carried via Ca^{2+} channels similar to the T-type Ca^{2+} channels.

METHODS

Mice (mixed breed ICR strain supplied from Yuhan Research Center, older than 4 weeks) were used as donors of the unfertilized eggs. Eggs for whole cell recording were prepared by using the same methods introduced by Park et al, (1991).

The bathing solution contained (in mM): 125 NaCl; 6 KCl; 2 $MgCl_2$; 20 $CaCl_2$; 20 HEPES-NaOH (pH 7.4). Pipette solution contained (in mM): 140 KCl; 2 $MgCl_2$; 1 $CaCl_2$; 10 Ethylene glycol-bis (β-aminoethyl ether) N, N, N′, N′-tetraacetic acid (EGTA) and pH was adjusted to 7.2 with KOH at 35℃ (pCa 7~8). Ca^{2+} antagonists such as nifedipine (Sigma) and amiloride (Fluka) were added without changes in the composition of bathing solution. To make Ba^{2+} containing solution, Ca^{2+} was replaced with equimolar Ba^{2+}. The concentration of $CaCl_2$ in the bathing solution was increased up to 20 mM to make the membrane stable and to facilitate the giga-seal formation (Peres, 1987). Eggs of which cytoplasmic contents were squeezed into pipette were discarded. All eggs were used within 8 hours after collection from the oviduct. Whole cell currents were recorded at room temperature.

Membrane potential was held at −80 mV except when obtaining activation and inactivation curves in this experiment. A series of test potentials (TPs) were given in 10 mV steps from −50 mV to 50 mV for 200 ms~500 ms. The activation curve of the current was obtained by measuring the peak amplitude of the current in response to the various potentials going from −90 mV to 0 mV in 5 mV steps was measured (holding potential, $V_h = -100$ mV) and normalized to maximal current and plotted against the TPs. To obtain the inactivation curve, a two-step pulse with a 10 ms interval between prepulse (PP) and TP fixed at −10 mV was given. Prepulse (−90 mV to 0 mV in 5 mV steps) from V_h of −100 mV as 500 ms long and test pulse was 200 ms. The amplitudes of peak current, normalized to maximal current obtained by −90 mV prepulse, were plotted against PPs. Data for activation and inactivation curves were fitted into a smooth curves using the Boltzmann relations, $I/I_{max} = [1 + \exp(-(V-V_{1/2})/k)]^{-1}$ and $I/I_{max} = [1 + \exp(V-V_{1/2})/k]^{-1}$, respectively (Nowycky, 1991). Signals were digitized by A/D converter (Labmaster™ and TL-1-125, Axon) and stored in a personal computer. Leak currents were subtracted from all currents by using the P/N protocol (pCLAMP, version 5.51, Clampex, Axon). The mean value was shown with both the standard deviation (SD) and the number of observations in parentheses.

RESULTS

Voltage-dependent properties of Ca^{2+} currents

Typical Ca^{2+} currents (i_{Ca}) elicited by the de-

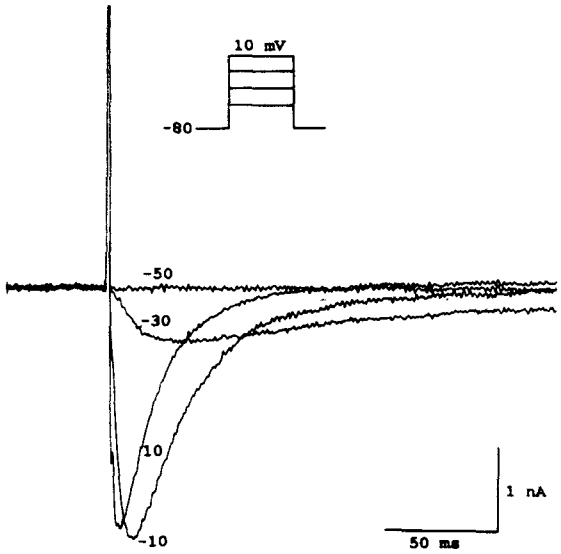

Fig. 1. Typical current traces, elicited by the step depolarization in mouse eggs.

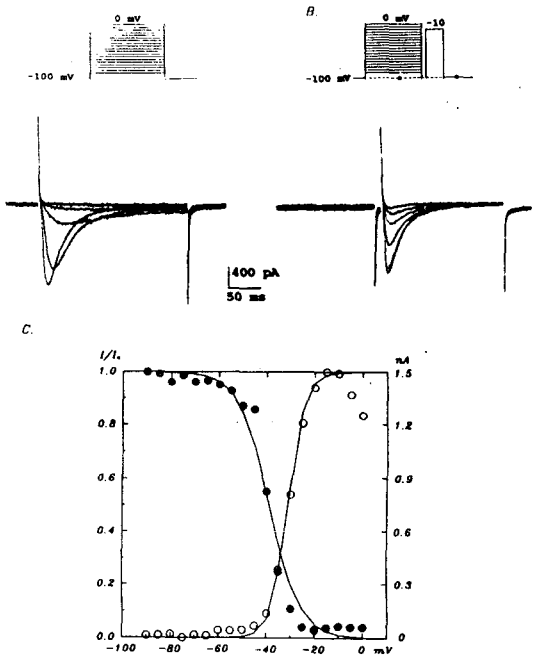

Fig. 2. Voltage-dependent activation and inactivation of Ca^{2+} currents in mouse eggs. A. the current traces evoked by depolarizing pulses from V_h=100 mV to various test pulses shown in the upper panel. B. the traces of current evoked by depolarizations to a fixed test potential of −10 mV from various prepulses. C. Activation and inactivation curves. Solid curves in C are the fitted ones derived from the Boltzmann relation. Data were obtained from peak current and normalized to their maximum elicited by the depolarization of −10 mV in A and by the prepulses of −90 mV in B. Open and closed circles represent data obtained from traces in A and B, respectively.

polarizing pulse increased up to the peak and then decayed rapidly in mouse eggs as shown in Fig. 1. These currents were activated in response to the depolarizing pulse of −50~+10 mV of −80 mV.

It can be seen from the current-voltage (I-V) relation that these currents appeared at potentials positive to −50 mV and were reversed at around 40 mV (see Fig. 3B). The currents reached a peak value within 11.8±3.26 ms (mean±S.D., n=21) of the onset of depolarization and the mean maximal amplitude was −2.26±0.71 nA (mean±S.D., n=21) at −10 mV. The inactivaton time constant, τ, was 28.2±9.59 ms (mean±S.D., n=21) at −10 mV. These observations suggest that i_{Ca} in the mouse egg was activated at a relatively low voltage and share many features with T-type currents (Fox, 1988).

To examine the voltage-dependency of i_{Ca} in the mouse egg, both the curve activation and inactivation were obtained from the currents recorded by using a series of step depolarization and two-step voltage pulses, respectively. This is shown in the upper panels of Fig. 2A and B.

During activation, the maximal current was obtained at −15 mV and peak currents, normalized to its maximum and plotted against TP, were fitted well with smooth curves derived from Boltzmann relation (using the software pClamp 5.51), $I/I_{max}=[1+\exp(-(V-V_{1/2})/k)]^{-1}$, where $V_{1/2}=-31$ mV and k=5 (open circle in Fig. 2C) in the steady-state inactivation curve, the peak currents, normalized to the maximal current obtained at PP of −90 mV, were fitted with the smooth curve, $I/I_{max}=[1+$

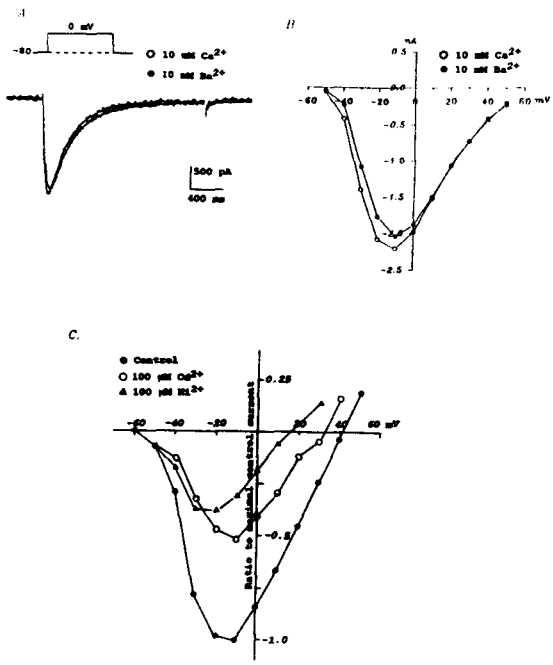

Fig. 3. Effect of divalent cations on i_{Ca}. A. the superimposed current traces recorded in the presence of Ca^{2+} and Ba^{2+}. B. I-V relations of i_{Ca} in a mouse egg. C, the effects of divalent Ca^{2+} channel blocker on I-V relations of i_{Ca} plotted as peak current in the presence of the divalent cations represented on each panel. Superimposed curves on each panel were obtained in same egg.

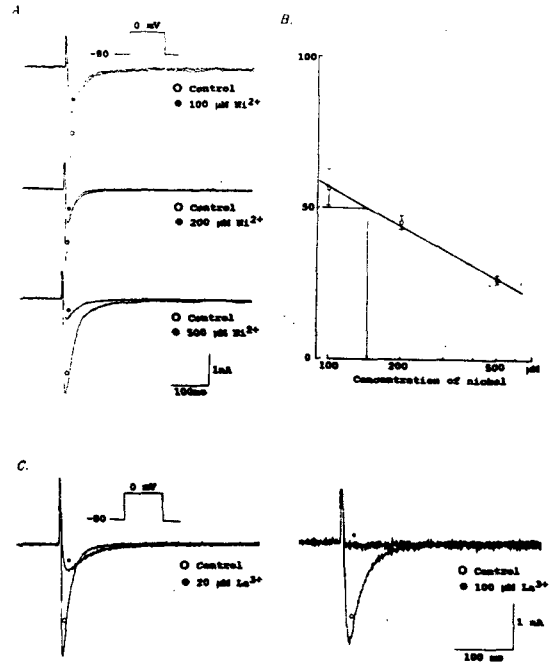

Fig. 4. Effect of Ni^{2+} and La^{3+} on i_{Ca}. A. superimposed current traces before and after the addition of Ni^{2+}. B. relation between the amount of decrease and Ni^{2+} concentration. Dose-response relation was well fitted with the first-order regression line (determinant coefficient, $\tau=0.995$) and the estimated half block (ED_{50}) of i_{Ca} was 160 μM Ni^{2+}. C. effect of La^{3+} on i_{Ca}. Reduced fraction (%) normalized to the control current peak was plotted against Ni^{2+} concentration ($[Ni^{2+}]_o$) in a log scale.

exp $(V-V_{1/2})/k]^{-1}$, where $V_{1/2}=-39$ mV and $k=6.5$ (closed circles in Fig. 2C). Figure 2C showed that i_{Ca} was activated at potentials more positive than -60 mV and inactivated in the range of -60 mV~-20 mV, indicating that both activation and inactivation of this current was steeply dependent on the low voltage.

Effects of divalent cations and nifedipine on Ca^{2+} currents

It was generally accepted that several pharmacological properties are a useful tool to divide Ca^{2+} channels into T-, N- and L-type (or P-type) as well as the voltage-dependence of the current, although their properties were used strictly as the criteria to classify the channel type. As the results obtained previously (in Fig. 2) imply that i_{Ca} in the mouse egg is carried via T-type channels, it was examined whether the pharmacological properties of Ca^{2+} channels in the mouse egg were compatible with those of the T-type channel. In these experiments, we observed the changes in i_{Ca} using three main criteria: comparison of the conductance between Ba^{2+} and Ca^{2+}; comparision of the inhibitory effect of Ni^{2+} and Cd^{2+}; and sensitivity of i_{Ca} to DHP antagonist (nifedipine).

As shown in Fig. 3A and B, the amplitude of

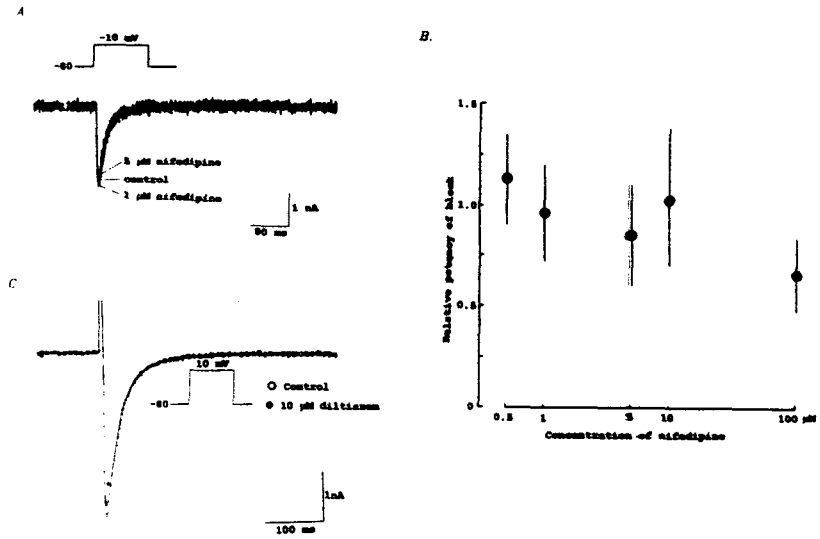

Fig. 5. Effect of nifedipine and diltiazem on Ca^{2+} current. A. current traces in response to does less than 5 μM. B. relation between the relative decrement of the current and nifedipine concentration. Current peak amplitudes, normalized to the peak of control current obtained at -10 mV, were plotted against the concentration of nifedipine. Closed circle and bar represent mean and standard deviation. C. effect diltiazem on i_{Ca}.

current was equal to or slightly reduced as compared with control current in the presence of Ba^{2+} substituted for Ca^{2+}. The I-V relations of the three currents were not different. Ni^{2+} in the range of 100~500 μM blocked i_{Ca} in a dose-dependent manner (ED_{50}=160 μM) (Figs. 4A and B). However, 100 μM Ni^{2+} was a more potent blockers of i_{Ca} than was equimolar Cd^{2+} (Fig. 3C).

Interestingly, i_{Ca} was reduced about 80% by adding 20 μM La^{3+}, known as the potent multivalent L-type channel antagonist, and was completely blocked in the presence of 100 μM La^{3+} (Fig. 4C).

Nifedipine, a dihydropyridine Ca^{2+} channel blocker, is known to block selectively L-type channels (Fox et al, 1987; Fox, 1988; Tsien et al, 1987; Nowycky, 1991). Ca^{2+} current in the mouse egg was not significantly decreased and sometimes even increased in the presence of nifedipine less than 10 μM (Fig. 5A). Even in 100 μM nifedipine, i_{Ca} was reduced only approximately by 35% (Fig. 5B). The current was insensitive to diltiazem, which is known to have an effect similar to that of nifedipine on L-type channels (Fig. 5C), These results indicate that the pharmacological properties of Ca^{2+} channels in the mouse egg are not identical to those of L-type, but closer to those of the T-type channel.

DISCUSSION

The present study was performed to classify the voltage-operated Ca^{2+} channels (VOCs) conducting i_{Ca} in mouse eggs according to the common criteria for T-, N-, or L- type channels (Fox et al, 1987; Llinás et al, 1992; Nowycky et al, 1985; Tsien et al., 1988; Tsien & Tsien, 1990). Inward currents evoked by depolarizing pulses in mouse eggs were regarded as i_{Ca} on the basis of following reports or results. First, inward current was increased as external $[Ca^{2+}]_0$ increased (Okamoto et al., 1977; Peres, 1987; Yoshida, 1985) and was blocked by divalent

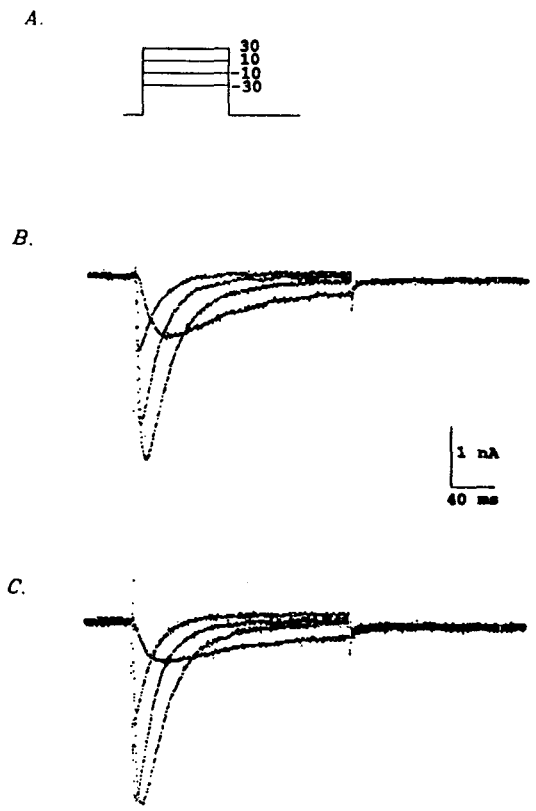

Fig. 6. Effect of TMA on i_{Ca}. A. pulse protocol B. current traces elicited by depolarization in a control bathing medium. C. current traces recorded in a bathing solution containing tetramethylammonium chloride (TMA-Cl). NaCl was replaced by the equimolar TMA-Cl. These traces were observed in the same egg.

cations such as Co^{2+}, Mg^{2+}, and Mn^{2+}. Secondly, the currents were carried selectively by Ca^{2+} as compared to other ions, such as Na^+, in the bathing solution (Yoshida, 1983; Yoshida, 1985). Finally, they were not affected in Na^+ free medium by replacing NaCl with tetramethylammonium chloride (TMA-Cl) (Fig. 6).

VOCs could be roughly divided into two groups according to the voltage range of activation and inactivation of channels (Kostyuk et al, 1988), e.g., LVA (low-voltage activated) channels like T-type and HVA (high-voltage activated) including L-, N-, and P-types (Llinás et al, 1992; Tsien & Tsien, 1990). Voltage dependency is not an absolute criterion to discriminate between the types of Ca^{2+} channel as the activation of L-type current becomes significant near at -30 mV in heart cells, and near at -10 mV in sensory neurons (Bean, 1985; Fox et al, 1987). In terms of the voltage range of kinetics and τ value, i_{Ca} in mouse eggs seems not to be carried by HVA channels (shown in Fig. 2). Since the current began to activate at a potential to approximately -40 mV and inactivated at -55 mV. Inactivation rate was rapid (τ was 28.2 ms at -10 mV). Since the most of HVA channel found in the sensory neurons in the chick and mouse become activated at least beyond -10 mV and their τ values range from 50 ms to 500 ms (Fox et al, 1987; Kostyuk et al, 1988; Tsien et al, 1988), they are dissimilar to the Ca^{2+} channels in mouse eggs. This is supported by evidence in the present study: (1) Ba^{2+} conductance was almost equal or even smaller than Ca^{2+} conductance; (2) The inhibitory effect of Ni^{2+} was greater than of Cd^{2+}; (3) Ca^{2+} currents in eggs were not significantly reduced by nifedipine. In our preliminary study and elsewhere, i_{Ca} in the mouse egg was neither altered in the presence of Bay K 8644, nor decreased significantly by adding diltiazem, known to a specific L-type channel blocker. These results indicate that the channels conducting Ca^{2+} in mouse eggs are similar to HVA channels, but share properties with the LVA, T-type channel. However, further study, including single channel kinetics is needed to confirm this hypothesis.

An unexpected result in this study was the finding that La^{3+} blocked Ca^{2+} currents more potently than other divalent blockers, e.g., Ni^{2+} and Cd^{2+}. Since La^{3+} was known as the dominant L-type current blocker, the amount of inhibition in i_{Ca} should have been less in La^{3+} than in Ni^{2+}, if the Ca^{2+} channels in mouse eggs were T-type. Unlike our expectations, current amplitude was reduced by 80% even in the presence of 20 μM La^{3+}, but a higher concentra-

tion of Cd^{2+} was required ($ED_{50}=160\,\mu M$). These findings are worthy to note with care. Because La^{3+} has multiple actions besides of inhibiting Ca^{2+} channels: La^{3+} displaces Ca^{2+} from the plasma membrane by binding to the La^{3+}-accessible site on the outside of the cells (Langer, 1982) and inhibits the plasmalemmal Ca^{2+}-pump (Sarkadi et al, 1977). If La^{3+} inhibits its Ca^{2+}-pump activity in the mouse egg, $[Ca^{2+}]_i$ could increase as the extrusion of Ca^{2+} is reduced. Also, if Ca^{2+} channels are inactivated by high $[Ca^{2+}]_i$, the decrease in current might have been greater due to a dual effect of La^{3+} both upon the channel and the Ca^{2+}-pump. The possibility that inhibition of pump activity by La^{3+} contributed to current decrease could be excluded. Since the inactivation of i_{Ca} in the mouse egg was voltage-dependent (Hong et al, 1991; Park et al, 1991), Therefore, the potent blocking effect of La^{3+} on Ca^{2+} channels in mouse egg may be due to the displacement of Ca^{2+} by impermeant La^{3+} on the outer portion of channel.

Physiological function of T-type channels is thought to generate pacemaker activity, while that of L-type to be involved in excitation-response coulpling and Ca^{2+} influx (Tsien et al, 1988; Tsien & Tsien, 1990). T-type channels are found frequently in cardiac pacemaker cells, Aplysia bag cells, and in certain vascular smooth muscle cells (Fox et al, 1987; Hagiwara et al, 1988; Sturek & Hermsmeyer, 1986). In contrast to T-type channel distribution, L-type channels are found in motor nerve terminals, synaptosomes, and growth cones of sympathetic neurons (Lipscombe et al, 1988; Reynolds et al, 1986; Thayer et al, 1988). Considering that each type of channels plays relatively specific functions, T-type channel in the mouse eggs may be used to generate and trigger Ca^{2+}-induced Ca^{2+} release (CICR) contributing to the rise in Ca^{2+} observed during fertilization (Cuthbertson et al, 1981; Miyazaki, 1991). This assumption is supported by the fact that $[Ca^{2+}]_i$ is increased by the electrical stimulation inducing parthenogenesis and that the elevation of $[Ca^{2+}]_o$ facilitated the increase in $[Ca^{2+}]_i$ in oocytes of mouse and rabbit (Fissore & Robl, 1992; Rickords & White, 1992).

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