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Regulatory Action of β-adrenergic Agonist and 8-bromocyclic AMP on Calcium Currents in the Unfertilized Mouse Eggs

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= ABSTRACT=

There are many reports suggesting that calcium influx and intracellular calcium concentration ($[Ca^{2+}]_i$) are related to cell signalling in various cells. However, it has not been reported that calcium channel activation is affected by the substances involved in signal transduction pathways in the mouse eggs. In this study, the effects of isoprenaline (ISP) and cyclic AMP on calcium influx through calcium channels were investigated to show their relationship with the signal transduction process in unfertilized mouse eggs. Using whole cell voltage clamp techniques, calcium currents, elicited by the depolarizing pulses of 300 ms duration (from − 50 mV to 50 mV in 10 mV increments) from a holding potential of − 80 mV, were recorded. The current-voltage (I-V) relation of calcium currents was shown to be bell-shaped; the current began to activate at − 50 mV and reached its maximum (− 1.33±0.16 nA: mean±S.E., n= 7) at − 10 mV, then decayed at around 50 mV. Calcium currents were fully activated within 7 ms~20 ms and completely inactivated 200 ms after onset of the step pulse.

ISP within the concentration ranges of 10^{-8} M~10^{-4} M dose-dependently increased the amplitude of the calcium current. The permeable cyclic AMP analogue, 8-bromocyclic AMP, also increased its maximal amplitude by 46% at 10^{-5} M, while protein kinase inhibitor (PKI), which is known to inhibit 0.02 phosphorylating units of cyclic AMP-dependent protein kinase (PKA) per microgram, decreased calcium currents. Currents recorded in the presence of PKI were resistant to increase by the application of 10^{-5} M. Also, PKI inhibited the calcium current increase elicited by ISP treatment.

These results suggest that β-adrenergic regulation of the calcium channel is mediated by the cAMP-dependent protein kinase. This signal transduction pathway might play a role in regulating $[Ca^{2+}]_i$ level due to the increase of calcium influx in mouse eggs.

Key Words: Mouse eggs, Calcium channel, Isoprenaline, 8-bromocyclic AMP, Protein kinase inhibitor, Whole cell voltage clamp technique.

INTRODUCTION

Changes in intracellular calcium concentration ($[Ca^{2+}]_i$) are important in the regulation of a variety of cellular functions, including secretion, contraction and hormone actions. It is known that increases in $[Ca^{2+}]_i$ in excitable cells arise mainly via voltage-activated calcium channels, whereas in non-excitable cells release of intracellular calcium by the second messenger inositol trisphosphate (IP_3) is prominent

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(Berridge, 1987). In addition to this typically transient increase in $[Ca^{2+}]_i$, which is independent of extracellular calcium, many non-excitable cells show a sustained phase of elevated $[Ca^{2+}]_i$ due to influx of extracellular calcium (Meldolesi & Pozzan, 1987; Merritt & Rink, 1987). In hamster eggs, a series of repetitive rises in $[Ca^{2+}]_i$ occurs throughout the whole cytoplasm of the egg at fertilization (Miyazaki, 1988), regulated by GTP, IP_3, and protein kinase C (Miyazaki, 1988; 1991; Miyazaki et al, 1990). Mouse eggs microinjected with acquorin have shown oscillatory $[Ca^{2+}]_i$ transients following exposure to mouse sperm (Cuthbertson, Whittingham & Cobbold, 1981), but the regulatory mechanism of $[Ca^{2+}]_i$ was not studied. However, forskolin was shown to elevate cyclic AMP in the oocyte and to inhibit germinal vesicle break down (Schultz et al, 1983; Urner et al, 1983). Moreover, a naturally occuring decrease in mouse oocyte cyclic AMP occurs during maturation (Schultz et al, 1983; Vivarelli et al, 1983) and correlates with the period during which oocytes become committed to resume meiosis (Schultz et al, 1983). These reports strongly suggest that cyclic AMP regulates mouse oocyte maturation.

In this paper we examined the effects of isoprenaline, 8-bromocyclic AMP and protein kinase inhibitor on the calcium currents of unfertilized mouse eggs. We report here the presense of a signal transduction pathway including cAMP and protein kinase A in unfertilized mouse eggs, investigated by whole cell voltage clamp technique.

METHODS

Egg donors

Mature virgin female golden hamsters, maintained under controlled lighting (8 h dark/16 h light), were injected I.P. with 5 i.u. of pregnant mare serum gonadotropin (Sigma) in the early morning. Forty-eight hours later, they were injected I.P. with 5 i.u. human chorionic gonadotropin (Sigma). The mice were killed, the oviducts opened and the eggs removed 15~18 h after the last injection.

Pre-treatment of eggs

All eggs were removed from the oviduct in a solution containing (mM): NaCl, 125; KCl, 6; $CaCl_2$, 2; $MgCl_2$, 1.2; HEPES, 20. This medium, referred to as standard solution in this paper, had a pH of 7.4 and contained polyvinylpyrrolidone (2 mg/ml; Sigma).

To remove the cumulus oophrous each egg was incubated for 2~4 min in standard solution containing hyaluronidase (1 mg/ml; type I-S, Sigma). To remove the zona pellucida each egg, freed from cumulus, was bathed for 1~3 min in standard solution containing protease (10 unit: type VII, Sigma). The enzyme treatments were carried out at room temperature (22~25℃).

Solutions

The solution used to superfuse eggs contained (mM): NaCl, 100; Na-pyruvate, 0.1; Na-lactate, 10; $MgCl_2$, 1.2; $CaCl_2$, 10; HEPES, 20; TEA-Cl, 20; adjusted to pH 7.4 with NaOH. The internal solution of the patch electrode normally contained (mM): CsCl, 115; ATP, 1; di-tris creatine phosphate, 5; $MgCl_2$, 2; TEA-Cl, 20; EGTA, 10; NaCl, 5; adjusted pH to 7.4 with CsOH. In some case, 1 ml internal solution contained: 0.2 mg protein kinase inhibitor (PKI), which was known to inhibit 0.02 phosphorylating unit of cyclic AMP-dependent protein kinase (PKA) per microgram. During experiments, cells were superfused (1 ml/min) at room temperature(22~25℃).

Current recording

The cells were voltage-clamped by using a whole cell patch clamp apparatus (Nihon Koden, CEZ-2100) according to the original technique developed by Hamil et al. (1981). Glass electrodes with resistances of 2~3 MΩ were used. The data were recorded on the computer hard disc through A/D & D/A converter

(Axon, AXOLAB 1100) for future analysis. Data were displayed on a digital oscilloscope (Kenwood, CS-8010).

Stimulation, data acquisition and analysis were performed with pClamp software (Axon, pClamp 5.51).

RESULTS

Isolation of the calcium current

For isolation of the calcium current in mouse eggs, the outward current was inhibited by the substitution K^+ with Cs^+ in the internal solution and TEA^+ on both sides of the cell membrane. A sodium channel blocker was not used because the channel is reported not to appear until the 8-cell stage (Hagiwara, 1983; Hagiwara & Jafe, 1979; Mitani, 1985).

We recorded the calcium current during depolarizations to various membrane potentials from a holding potential of −80 mV for 300 ms (Fig. 1A & 1B). The current appeared at potentials positive to −50 mV and was reversed at around 50 mV (Fig. 1C). The maximal amplitude was −1.33±0.16 nA (mean±S.D., n=7) at −10 mV.

Effects of isoprenaline on the calcium current

We examined the effect of isoprenaline in

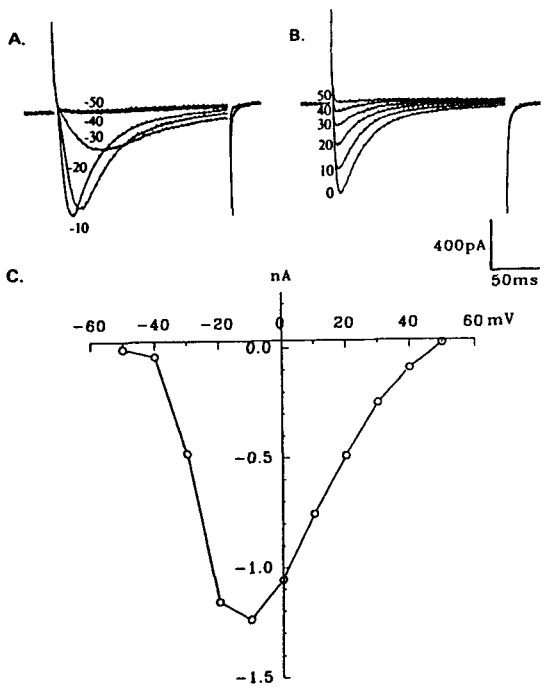

Fig. 1. Calcium currents and current-voltage relation in mouse eggs. A and B, current traces elicited by step depolarization of 300 ms duration from −50 mV to 50 mV with every 10 mV increment. Membrane potential was held at −80 mV. At −10 mV, current amplitude reached its maximum. C, current-voltage relation of Ca^{2+} current.

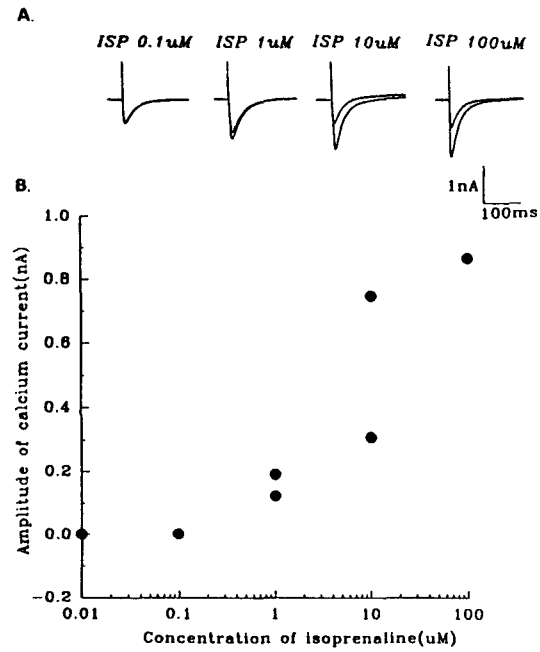

Fig. 2. Dose-response curve for the effect of isoprenaline (ISP) on the Ca^{2+} current. A, ISP dose-dependently increased current amplitude. Current traces in upper panel were rocorded in the same egg. B, Dose-response curve. abscissa: micromolar concentration of isoprenaline in log scale, ordinate: difference of amplitude between current maximum in ISP-free solution and that in the solution containing ISP, Current maximum was obtained from the current trace in response to −10 mV from holding potential of −80 mV.

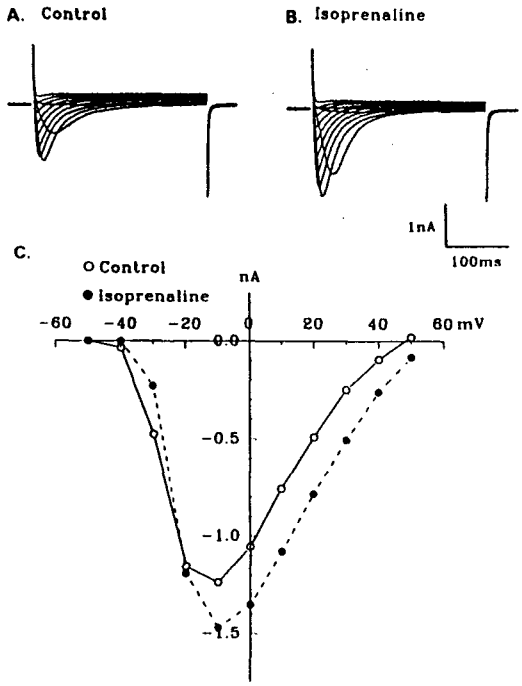

Fig. 3. Isoprenaline effect on Ca^{2+} currents. Currents were increased by the application of 10^{-5} M ISP (A and B). C, current-voltage relation before (○) and after (●) application of ISP.

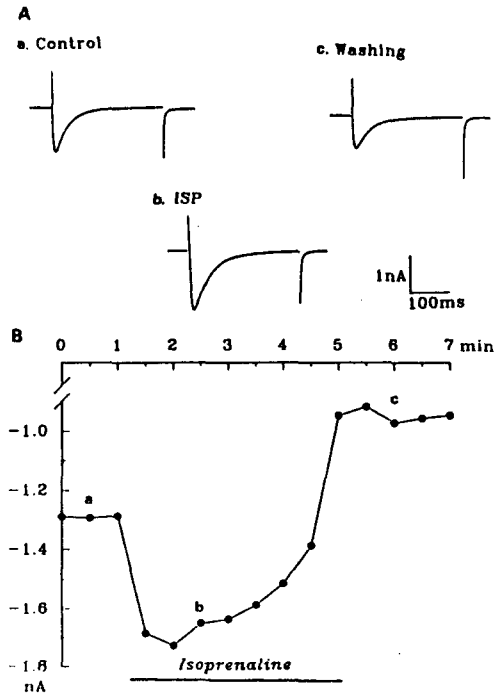

Fig. 4. Reversible effect of ISP on Ca^{2+} current. A, Ca^{2+} current increased in the presence of ISP (b) and recovered after washing out ISP (c). B, Change of Ca^{2+} current amplitudes for the ISP application. During ISP stimulation for 5 min, current magnitude was increased ans 2 min later decreased slowly. After substitution of ISP-free solution (c), current size was significantly smaller than that in (b) and even in (a).

order to investigate the existence of a signal transduction pathway including cAMP and cAMP-dependent protein kinase. Isoprenaline has been known to increase cAMP via GTP-binding proteins (Irisawa & Kokubun, 1983; Reuter, 1983; 1987; Siegelbaum & Tsien, 1983; Tsien, 1983). Potentiation of calcium current by β-adrenergic stimulation was examined quantitatively with various concentrations of isoprenaline (Fig. 2). Isoprenaline increased calcium current in a dose-dependent manner at all test potentials where calcium current was activated. For example, the amplitude of peak calcium current at -10 mV was increased 1.5-fold by 10 μM isoprenaline. The current-voltage relations in the control and with 10 μM isoprenaline are illustrated in Fig. 3. The threshold dose of isoprenaline for increasing calcium current was 1 μM. The calcium current was increased without a change in the apparent reversal potential (Fig. 3), time to peak and inactivation time constant (Fig. 5). Time course of calcium current produced by superfused isoprenaline (Fig. 4). The amplitude of calcium current was measured with a holding potential of -80 mV and test potential of -10 mV. During the solid line, superfusion of cell with 10 μM isoprenaline was performed. An increase of calcium current appeared 30s after the start of perfusion and was maximal at 1 min. Examples of the current record are shown above the graph (a-c) for the time indicated in the graph (Fig. 4).

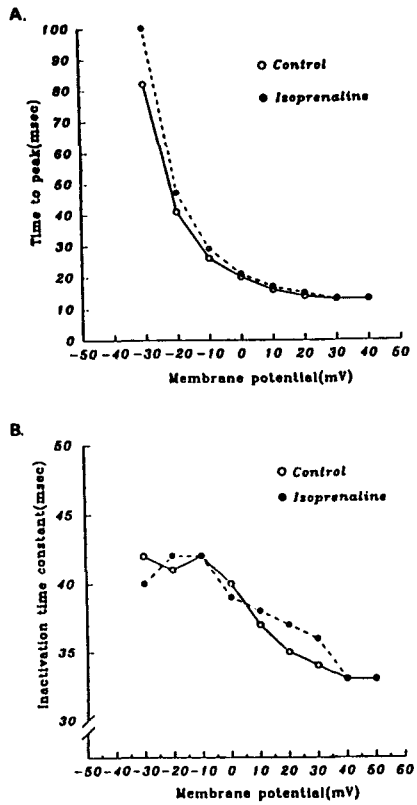

Fig. 5. The effect of ISP on both time to peak (A) and inactivation time constant (B) of the Ca^{2+} current. Open circles and closed ones represent the values obtained before and after application of ISP, respectively.

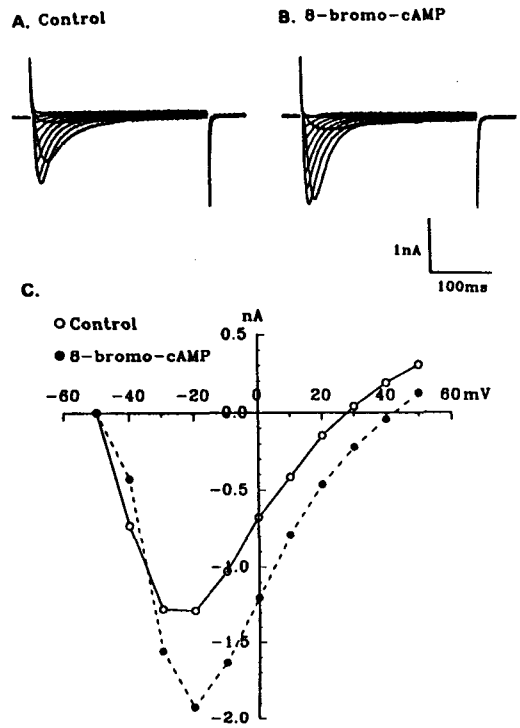

Fig. 6. Effect of 8-bromocyclic AMP on the Ca^{2+} currents recorded in the same egg. Current traces (A) and current-voltage relation (B) before (○) and after (●) 8-bromocyclic AMP (10^{-5} M) application. Extracellular 8-bromocyclic AMP increased Ca^{2+} currents.

Effects of 8-bromocyclic AMP on the calcium current

We have analysed the effect of 8-bromocyclic AMP on calcium channels. The 8-bromocyclic AMP, a membrane permeable analogue of cAMP, has been shown to increase the amplitude of the calcium current via activation of cyclic AMP-dependent protein kinase (Bean et al, 1984; Cachelin et al, 1983).

When 8-bromocyclic AMP was applied extracellularly, the nucleotide increased the membrane currents in a similar manner to isoprenaline. The amplitude of calcium current was increased by 46%(Fig. 6A, 6B). The current-voltage relation in the control and with 10 μM 8-bromocyclic AMP are show in figure 6C. The calcium current was reversibly increased by 8-bromocyclic AMP without a change of time to peak and inactivation time constant (Fig. 7 & 8). The amplitude of calcium current was measured with holding potential of −80 mV and test potential of −10 mV. During the solid line, superfusion of the cell with 10 μM 8-bromocyclic AMP was performed. An in-

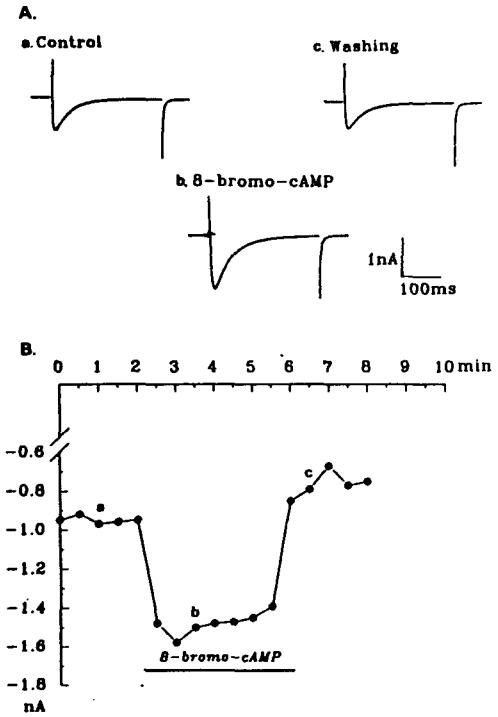

Fig. 7. Reversible effect of 8-bromocyclic AMP on Ca^{2+} current. A, Ca^{2+} current increased in the presence of 8-bromocyclic AMP in bathing solution (b) and recovered after removal of 8-bromocyclic AMP (c). B, Change of Ca^{2+} current amplitude for the ISP application with time. Current was increased for 5 min of 8-bromocyclic AMP application (b) and recovered to control level after washing out the 8-bromocyclic AMP (c). Dots indicated the current maximum obtained from the current elicited by step depolarization of −10 mV from −80 mV at 30 sec intervals.

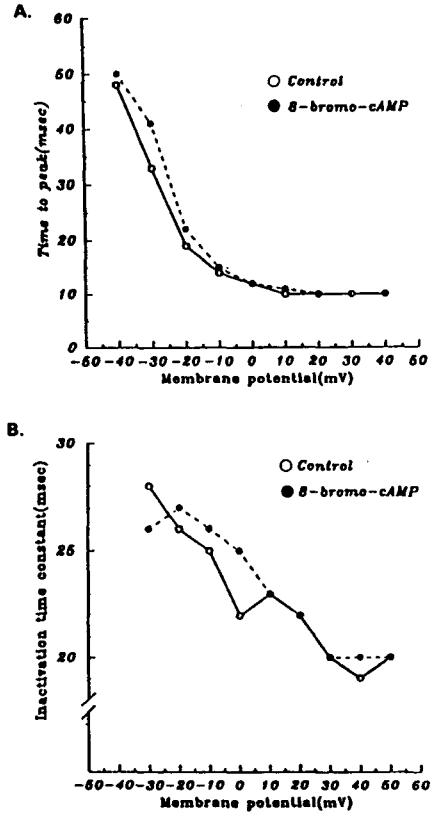

Fig. 8. The effect of ISP on both time to peak (A) and inactivation time constant (B) of the Ca^{2+} current. Open circles and closed ones represent the values obtained before and after application of 8-bromocyclic AMP, respectively.

crease of calcium current appeared 30 s after the start of perfusion and was maximal at 1 min. Examples of the current record are shown above the graph (a-c) for the time indicated in the graph (Fig. 7).

Effects of protein kinase inhibitor

We examined the effects of β-adrenergic agonist and cyclic AMP in order to investigate whether the phosphorylation of calcium channel is involved.

When protein kinase inhibitor was applied intracellularly by perfusion of the pipette, the calcium current was inhibitted (Fig. 9).

Effects of protein kinase inhibitor and isoprenaline

In order to investigate whether calcium channel regulation is affected by the substances involved in signal transduction pathways, the effects of a β-adrenergic agonist and a protein kinase inhibitor were examined. With isoprenaline and cyclic AMP we found a significant increase in the amplitude of the calcium current.

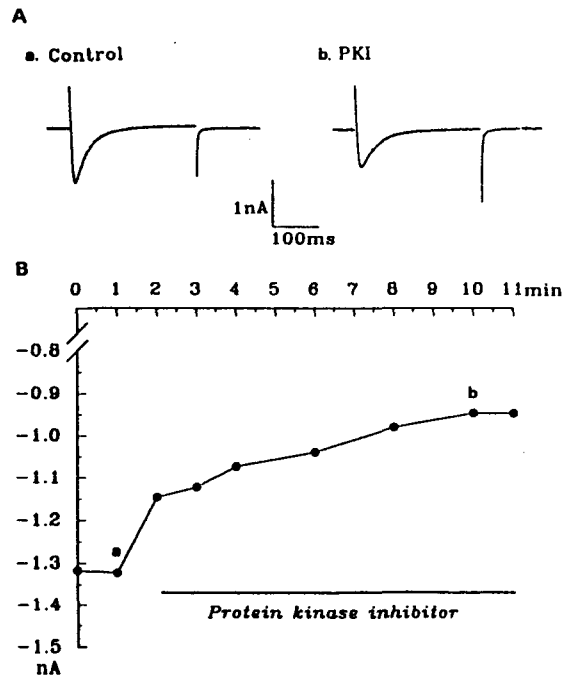

Fig. 9. Inhibitory effect of protein kinase inhibitor (PKI) on the Ca^{2+} current. A, Intracellular PKI perfusion suppressed current activation. B, Inhibitory effect of PKI with time. Currents were measured with 1 or 2 min intervals and the voltage protocol was the same as that of Fig. 7.

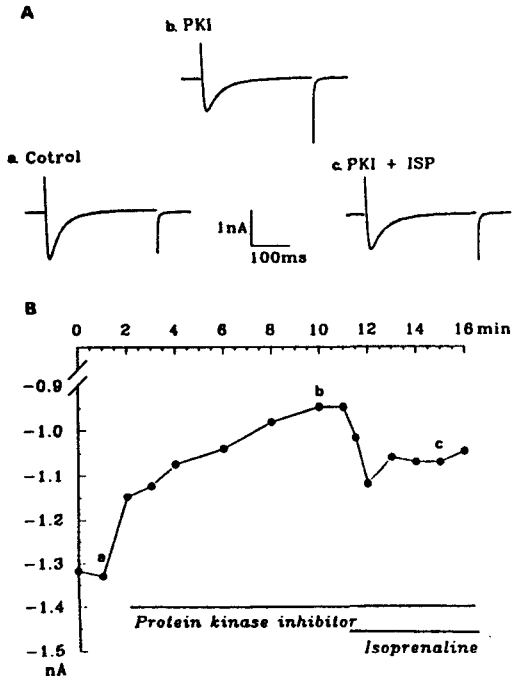

Fig. 10. Decrease of ISP effect in the presence of PKI. A and B, Ca^{2+} currents recorded whilst perfusing PKI into patch pipette were not significantly increased by the ISP treatment (c). Both the interval between current recordings and voltage protocol were the same as those in Fig. 7. and Fig. 9.

However, the calcium current recorded in the presence of protein kinase inhibitor were resistant to increase by the application of 10 μM isoprenaline (Fig. 10). Also the protein kinase inhibitor suppressed the increase of calcium current elicited by isoprenaline application (Fig. 11).

DISCUSSION

The main finding in the present study is that isoprenaline and 8-bromocyclic AMP increase the calcium current in a dose-dependent manner without change of the current-voltage relation and inactivation time constant (Fig. 2, 3, 5, 6). These results suggest that isoprenaline and 8-bromocyclic AMP modulate calcium channel without activation of other channels or change of kinetics in mouse eggs. Both isoprenaline and 8-bromocyclic AMP increase cyclic AMP within cell, which presumably causes phosphorylation of the calcium channel or of a protein closely associated with it (Reuter, 1979; Tsien & Siegelbaum, 1978). In addition, the protein kinase inhibitor decreased the calcium current (Fig. 9, 11) and suppressed the calcium current increase elicited by isoprenaline application (Fig. 11). These results suggest that the calcium channel in the unfertilized mouse egg is modulated by cascade reactions underlying β-adrenergic stimulation, as found by Reuter

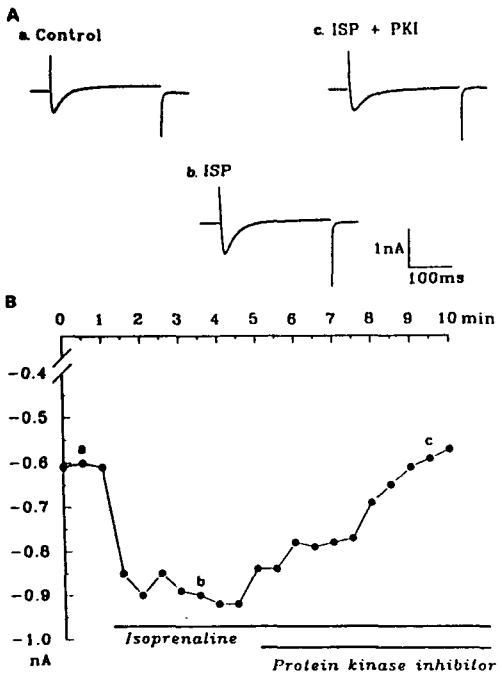

Fig. 11. *Inhibitory action of PKI on ISP effect. A, current traces in control state (a), in bathing solution containing ISP (b) and whilst adding PKI inside the egg, and applying extracellular ISP, simultaneously (c). B, Suppressive effect of PKI on the Ca^{2+} current increase evoked by ISP application. Current increased by ISP stimulation was gradually depressed as the perfusion of PKI continued.*

(1983).

In the present study, the decrease in calcium current with the lapse of time is a matter for consideration. This phenomenon has an effect on verification of the effects on isoprenaline, cyclic AMP and protein kinase inhibitor. At first, the calcium currents increased with isoprenaline superfusion, then the current successively decreased with sustained isoprenaline superfusion (Fig. 4B). This appearance could result from 1) decrease of driving force for calcium influx, 2) the depletion of substrates for the calcium channel and 3) the rundown phenomenon (Hille, 1992). The calcium current decreses regularly with the passage of time in most eggs. So, the phenomenon was thought to be due to the rundown of calcium current. Also, the effect of the protein kinase inhibitor on the calcium current suggests a rundown phenomenon. But, protein kinase inhibitor suppressed the calcium current increase elicited by isoprenaline superfusion. Therefore, the effect of protein kinase inhibitor is thought to be an inhibitory effect on the calcium current. Also, the effects of isoprenaline deserve attention. Isoprenaline increases calcium current not only through an increase in intracellular cyclic AMP (Irisawa & Kokubun, 1983; Reuter, 1983; 1987; Sigelbaum & Tsien, 1983; Tsien, 1983) but also via direct regulation of calcium channels (Brown & Birnbaumer, 1988; Imoto et al, 1988; Nahorski, 1990; Yatani et al, 1987). If the calcium current increase results only from the cyclic AMP-protein kinase cascade, isoprenaline should not increase the calcium current under whilst perfused with protein kinase inhibitor. But, isoprenaline slightly potentiated calcium currents in the presence of the protein kinase inhibitor (Fig. 10B). Therefore, we can not completely exclude the possibility of direct activation of G-proteins by isoprenaline.

We found that isoprenaline and 8-bromocyclic AMP increase the calcium current and protein kinase inhibitor decreases the calcium current in unfertilized mouse eggs. Our results suggest that protein phosphorylation by PKA play an important role in the regulation of $[Ca^{2+}]_i$ in the unfertilized mouse eggs.

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