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The Role of Intracellular Mg^{2+} in Regulation of Ca^{2+}-activated K^+ Channel in Pulmonary Arterial Smooth Muscle Cells of the Rabbit

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Although the Ca^{2+}-activated K^+ ($I_{K,Ca}$) channel is known to play an important role in the maintenance of resting membrane potential, the regulation of the channel in physiological condition is not completely understood in vascular myocytes. In this study, we investigated the role of cytoplasmic Mg^{2+} on the regulation of $I_{K,Ca}$ channel in pulmonary arterial myocytes of the rabbit using the inside-out patch clamp technique. Mg^{2+} increased open probability (Po), but decreased the magnitude of single channel current. Mg^{2+}-induced block of unitary current showed strong voltage dependence but increase of Po by Mg^{2+} was not dependent on the membrane potential. The apparent effect of Mg^{2+} might, thus, depend on the proportion between opposite effects on the Po and on the conductance of $I_{K,Ca}$ channel. In low concentration of cytoplasmic Ca^{2+}, Mg^{2+} increased $I_{K,Ca}$ by mainly enhancement of Po. However, at very high concentration of cytoplasmic Ca^{2+}, such as pCa 5.5, Mg^{2+} decreased $I_{K,Ca}$ through the inhibition of unitary current. Moreover, Mg^{2+} could activate the channel even in the absence of Ca^{2+}. Mg^{2+} might, therefore, partly contribute to the opening of $I_{K,Ca}$ channel in resting membrane potential. This phenomenon might explain why $I_{K,Ca}$ contributes to the resting membrane potential where membrane potential and concentration of free Ca^{2+} are very low.

Key Words: Ca^{2+}-activated K^+ channel, Mg^{2+}, Pulmonary arterial smooth muscle, Open probability, Unitary conductance

INTRODUCTION

Ca^{2+}-activated K^+ ($I_{K,Ca}$) channel is densely distributed in various types of cells and it has been proposed by many studies that it plays an important role in regulating resting membrane potential of smooth muscle cells (for review see Nelson & Quayle, 1995), repolarization of pacemaking neuronal cells (Pennefather et al, 1985), and regulation of intracellular Ca^{2+} (Brayden & Nelson, 1992; Miller et al, 1993; Leblanc et al, 1994). Biophysical properties of the channel have also been widely investigated (Levitan, 1988; Rudy, 1988; Latorre et al, 1989; McManus, 1991; Garcia et al, 1991; Toro and Stefani, 1991), but the regulation of the channel in physiological condition is not completely understood.

It was noticed that the single channel conductance recorded in cell-attached patch was significantly smaller compared with that recorded in inside-out patch, suggesting an existence of blockers inside the cell. It has been shown that intracellular Mg^{2+} and Na^+ act as natural inhibitors of $I_{K,Ca}$ channel (Morales et al, 1996; Snetkov et al, 1996). The kinetics of blockade showed a flickering nature, which led to shortening of mean open time and reduction of apparent unit current amplitude (Methfessel & Boheim, 1983; Moczydlowski & Latorre, 1983; McManus & Magleby, 1988). This type of blockade suggests that blocking ions reversibly bind to the open-state K^+ channel with fast kinetics. The aim of the present study is to investigate the possibility that blocking effect of Mg^{2+}

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is affected by the change of cytoplasmic Ca^{2+} concentration. We recorded the single channel activities of $I_{K,Ca}$ from smooth muscle cells of rabbit pulmonary and ear arteries, and the results demonstrate that Mg^{2+} not only blocks $I_{K,Ca}$ channel, but also can activate the channel when the cytoplasmic concentration of Ca^{2+} is low.

METHODS

Cell preparation

Isolated pulmonary arterial cells were prepared according to the method described previously (Lee et al, 1991). Main pulmonary artery and its primary branches were obtained from the rabbits (1～1.5 kg) which anaesthetized by intravenous injection of pentobarbital (50 mg/kg) and heparin (1000 IU/kg). The arterial tissue was dissected free of the surrounding adventitia and connective tissue, and cut into small pieces of 2～3 mm widths. The pieces were bathed in a Ca^{2+}-free normal Tyrode solution at 37°C for 10～15 min, and then incubated in Ca^{2+}-free solution containing collagenase (Worthington, 0.4 mg/ml), elastase (Sigma type II-A) and dithiothreitol (Sigma, 1.2 mg/ml) for 25 min. To obtain dispersed single cells, the digested fragments were gently agitated with large-bored pipette in KB solution. The dispersed cells were kept at 4°C in KB solution and used in 24 hours.

Solutions

Normal Tyrode solution contained (in mM): NaCl, 143; KCl, 5.4; $CaCl_2$ 1.8; $MgCl_2$ 1; glucose 5.5; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 5; buffered to 7.4 with NaOH. The pipette-filling solution contained (in mM): KCl, 150; HEPES, 5; ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid(EGTA), 5; adjusted pH to 7.4 with Trizma base (Tris, Sigma). The Ca^{2+}-free bath solution for inside-out patch contained (in mM): KCl, 150; HEPES, 5; EGTA, 5; $MgCl_2$, 0.5; buffered to 7.4 with Tris. For calculation of free Ca^{2+} and Mg^{2+} concentration, association constants among Ca^{2+}, Mg^{2+}, EGTA and ATP at pH 7.4 were taken from Fabiato & Fabiato (1979). All chemicals were obtained from SIGMA.

Electrophysiological recording and analysis of results

To record single channel currents, the inside-out patch clamp technique was used. High resistance seal more than 5 Gohm was formed between the borosilicate glass electrode (tip resistance of about 10 Mohm) and cell membrane by applying mild suction. Excised inside-out patches were made by withdrawing the pipette. Single channel currents were recorded with an Axopatch-1C amplifier, and fed to a pulse code modulator (Medical System) and to an A/D converter (Labmaster) simultaneously to store the data on the video tape recorder and on the computer. All the experiments were performed at room temperature, about 25°C.

RESULTS

Single channel activities were recorded in symmetrical K^+ concentrations (150 mM in both pipette and bath solutions) with the inside-out mode. The large-conductance $I_{K,Ca}$ channels were identified by their large conductance (>250 pS) and the increase of their channel openings by depolarization or an increase of intracellular Ca^{2+}. Fig. 1 showed the ensemble average currents obtained at various concentrations of cytoplasmic Ca^{2+} using the method developed by Carl & Sanders (1989). Voltage ramps from 0 mV to 100 mV for 2 seconds were applied in every 20 seconds

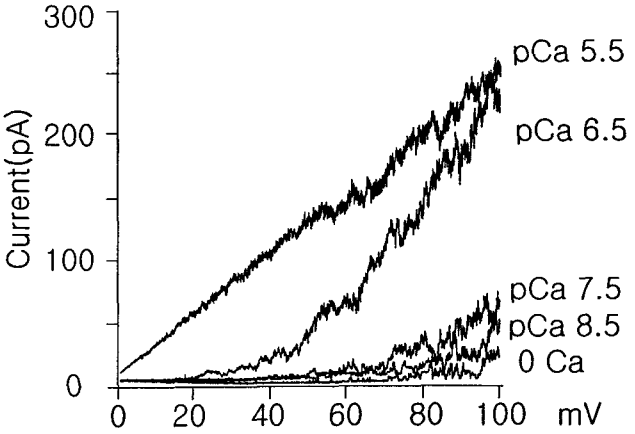

Fig. 1. The effect of intracellular Ca^{2+} on Ca^{2+}-activated K^+ current in pulmonary arterial smooth muscle cell. Voltage ramps from 0 mV to 100 mV for 2 seconds were applied in every 20 seconds and 15 current traces were averaged.

and 15 current traces were averaged. We regarded it as the typical voltage-dependent activation properties of the $I_{K,Ca}$ channels. As shown in Fig. 1, $I_{K,Ca}$ channels were completely open at pCa 5.5, whereas those were mostly in the close state at Ca^{2+}-free solution (<pCa 8.5) over the range of 0~+80 mV in a pulmonary arterial smooth muscle cell. The open state probability was heavily dependent on the cytoplasmic concentration of Ca^{2+}.

In order to test whether the effect of Mg^{2+} on $I_{K,Ca}$ depends on cytoplasmic Ca^{2+}, $I_{K,Ca}$ was observed at various concentrations of Ca^{2+}, in the absence and in the presence of 5 mM Mg^{2+}. The free Ca^{2+} and free Mg^{2+} concentrations were calculated by the computer program based on association constants between Ca^{2+}, Mg^{2+} and EGTA (Fabiato & Fabiato, 1979). The inside-out patch recordings in Fig. 2 showed the activity of $I_{K,Ca}$ channels in a patch containing 7 channels, where each current trace was drawn after the averaging of 15 current traces. 5 mM Mg^{2+} increased the magnitude of $I_{K,Ca}$ when concentration of cytoplasmic Ca^{2+} was below 0.1 μM, whereas it decreased the activity of $I_{K,Ca}$ channel at pCa 6.5. Under the condition of pCa 6, the effect was more complicated: increase at less positive potential, but decrease at more positive potential (>70 mV). This result suggests that the effect of Mg^{2+} depends not only on the cytoplasmic Ca^{2+}, but also on the membrane potential. In several patches, we obtained the same results.

Intracellular Mg^{2+} not only increased $I_{K,Ca}$ but also decreased it depending on $[Ca^{2+}]_i$ and membrane potential. To unravel the complicated action of Mg^{2+}, the effect of Mg^{2+} was observed at various potentials after fixing free cytoplasmic Ca^{2+} concentration at pCa 6 where $I_{K,Ca}$ channel response to Mg^{2+} was complex. Fig. 3A clearly showed that at pCa 6 the channel activity increased as membrane potential was

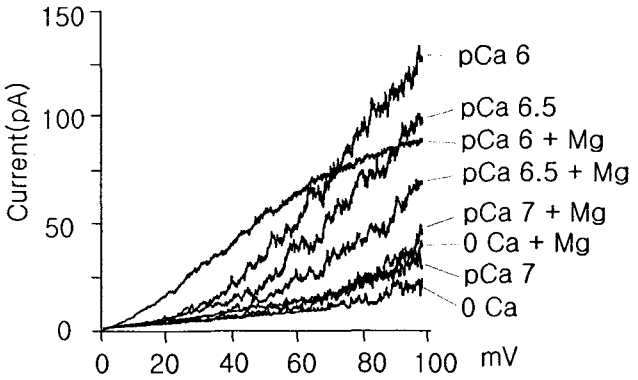

Fig. 2. The effect of intracellular Mg^{2+} on Ca^{2+}-activated K^+ current at the various concentrations of intracellular Ca^{2+}. Voltage ramps from 0 mV to 100 mV for 2 seconds were applied in every 20 seconds and 15 current traces were averaged. Each current trace was recorded from the same patch containing 7 channels.

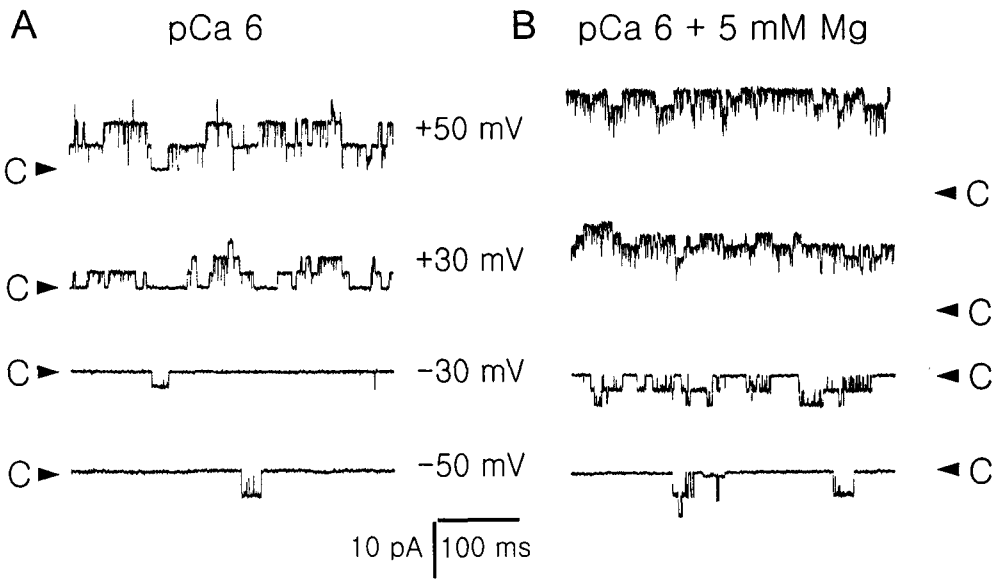

Fig. 3. Current traces of Ca^{2+}-activated K^+ channels at various holding potentials. A. Recording at pCa 6, control. B. Recording in the presence of intracellular 5 mM $MgCl_2$. Free Ca^{2+} was fixed at pCa 6 using 5 mM EGTA. C means the close state of all channels.

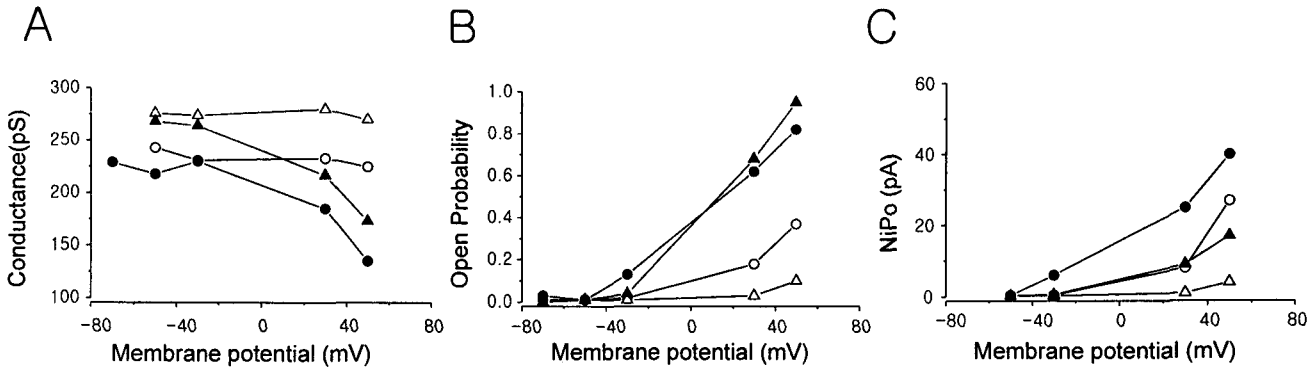

Fig. 4. The effect of intracellular 5 mM Mg^{2+} on the unit conductances and the open probabilities of Ca^{2+}-activated K^+ channels from pulmonary and ear arterial smooth muscle cells. A. Mg^{2+} reduced unitary conductances in depolarization range, but not in hyperpolarization range. B. Mg^{2+} increased open state probability. C. *NiPos* calculated from the representative data shown in Fig. 3. Circle: pulmonary arterial smooth muscle cell, triangle: ear arterial smooth muscle cell, open symbols: control data at pCa 6; filled symbols: after the application of 5 mM $MgCl_2$.

depolarized, which is a characteristic feature of $I_{K,Ca}$ channel. Addition of 5 mM Mg^{2+} greatly increased the open state probability of $I_{K,Ca}$ channels over the entire potential range (Fig. 3B). The effect of Mg^{2+} on the amplitude of unitary current was, however, varied depending on the membrane potential: the amplitude of inward current recorded at negative potential was not affected by cytoplasmic Mg^{2+}, whereas outward current was decreased by the further depolarization of membrane potential. Unitary conductance and open state probability measured from this record were plotted in Fig. 4. In Fig. 4A, the unitary conductance was shown to be decreased by the application of Mg^{2+}. The Mg^{2+}-induced block of unitary current revealed marked voltage dependence allowing $I_{K,Ca}$ channel inward rectification. The unitary conductance was decreased after the application of 5 mM Mg^{2+}, by 3~6% at -50~-30 mV, by 22% at +30 mV, and by 36% at +50 mV in this patch, respectively. In $I_{K,Ca}$ channel from ear artery we had the same results as shown in Fig. 4A (triangle). However, as shown in Fig. 4B, the Mg^{2+}-induced increase of Po exhibited weak voltage dependence, 50~70% at +30 mV and 60~75% at +50 mV both in pulmonary and ear arterial $I_{K,Ca}$ channels. The calculated *NiPos* from the data presented in Fig. 3, where N, i, and Po mean a number of channels, unitary current, and open state probability, were shown in Fig. 4C. Round circles and triangles are data from pulmonary and ear arterial smooth muscle cell of the rabbit. Current traces of single channel from ear arterial smooth muscle cells were not shown here. There is no difference

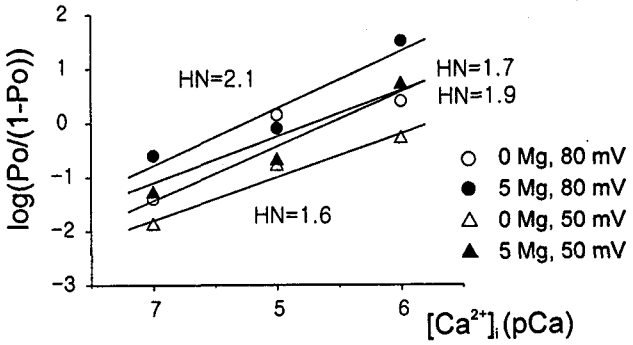

Fig. 5. A Hill plot of open state probability with respect to pCa. 5 mM Mg^{2+} increased Hill number from 1.7 to 2.1 at 80 mV(circle) and from 1.6 to 1.9 at 50 mV (triangle). HN means Hill number.

between two kinds of myocytes, suggesting that the $I_{K,Ca}$ channel modulation by Mg^{2+} is a general characteristic of $I_{K,Ca}$ channels. In 4 patches, we got the same results.

We investigated whether the effect of Mg^{2+} involves the interaction between Ca^{2+} and Ca^{2+} binding sites of $I_{K,Ca}$ channel. To estimate if the increase of open state probability by Mg^{2+} is due to the disclosure of new binding site for Ca^{2+}, Hill number was obtained and the representative data were shown in Fig. 5. Mg^{2+} slightly increased Hill number from 1.7 to 2.1 at 80 mV and from 1.6 to 1.9 at 50 mV, respectively.

DISCUSSION

In this study, Mg^{2+} increased open state probability, but decreased the magnitude of single channel current. Mg^{2+}-induced block of unitary current showed strong voltage dependence but open probability enhanced by Mg^{2+} did show weak voltage dependence. Therefore, the final composite effects of Mg^{2+} could be observed on ensemble average current of $I_{K,Ca}$ as shown in Fig. 2. The apparent effect of Mg^{2+} might depend on the proportion between opposite effects on the open probability and on the conductance of $I_{K,Ca}$ channels. In the case of pCa 6, the former effect prevails at membrane potential below 70 mV, and the latter effect prevails at the higher voltage range. In a low concentration of Ca^{2+}, Mg^{2+} increased $I_{K,Ca}$ by mainly enhancement of Po, but, at very high concentration of Ca^{2+}, such as pCa 5.5, Mg^{2+} decrease $I_{K,Ca}$ through the inhibition of unitary current. This illustration is consistent with the data in Fig. 2. Although the activity of $I_{K,Ca}$ channels recorded in inside-out patch was very low in the range of resting membrane potential in smooth muscle, there are many reports about the contribution of $I_{K,Ca}$ on the resting membrane potential (for review see Nelson & Quayle, 1995). Moreover, Mg^{2+} could activate the channel even in the absence of Ca^{2+}. These phenomena might explain why $I_{K,Ca}$ contribute to the resting membrane potential, where membrane potential and concentration of free Ca^{2+} are very low.

Data for the probability of opening, as a function of internal Ca^{2+} are usually fitted with the following equation:

$$Po=Po_{max}[Ca]^N/([Ca]^N + K_d),$$

where N and K_d is the Hill coefficient and the apparent dissociation constant. Po and Po_{max} are open probability and its maximum value at a given potential. This equation is based on the following reaction scheme (McManus, 1991):

$$\text{Closed channel} + NCa^{2+} \rightleftharpoons \text{Open channel-}Ca_N,$$

hence, N means the actual number of Ca^{2+} binding sites in the channel protein. It was previously suggested that full activation of $I_{K,Ca}$ channel requires the binding of more than two Ca^{2+} ions (McManus, 1991) but Hill coefficient as large as 5.3 has been reported by Carl & Sanders (1989). For the skeletal muscle $I_{K,Ca}$ channels, Golowasch et al. (1986) found a marked effect of internal Mg^{2+} on N. The Hill coefficient increases 2.1- to 3-fold when the internal Mg^{2+} is raised from 0 to 10 mM, and they suggested that Mg^{2+} appears to act allosterically by binding to a site with a divalent cation selectivity that is different from that of the Ca^{2+} binding site. Recent data of molecular cloning of *mslo* suggest that intracellular long C-terminus contains just 4 possible Ca^{2+} binding sites (Butler et al, 1993). According to our data in Fig. 5, Hill coefficient is about 2, implying that $I_{K,Ca}$ channel has more than two Ca^{2+} binding sites in itself. Mg^{2+} has a little effect on the Hill number. Therefore, Mg^{2+} does not appear to make new binding sites for Ca^{2+} in the $I_{K,Ca}$ channel of pulmonary arterial smooth muscle cell. It remains to be elucidated whether our data indicate that $I_{K,Ca}$ channel protein in pulmonary arterial cell is different from those of other tissues.

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