Activation of Ca^{2+}-activated K^{+} Channels by Beta Agonist in Rabbit Coronary Smooth Muscle Cells

Duck-Sun Ahn, Young-Ki Jeong, Young-Ho Lee and Bok-Soon Kang

Isoproterenol (ISO), a beta agonist, causes hyperpolarization of coronary smooth muscle cells via an increase in K^{+} conductance. This hyperpolarization may cause the coronary vasorelaxation by decreasing the cytoplasmic Ca^{2+} concentration. It is well known that the activation of beta adrenoceptors stimulates the adenylate cyclase activity, and the resulting K^{+} channel phosphorylation by cAMP-dependent protein kinase may be responsible for ISO-induced increase in K^{+} channel activity. However, it is not clear whether the increase in K^{+} channel activity by ISO is exclusively due to the activation of adenylate cyclase or not. In this research, the effect of ISO on the isometric tension and the mechanism of ISO-induced K^{+} channel activation were investigated in various patch clamp conditions. The summarized results are as follows. ISO- and pinacidil induced vasorelaxation was significantly inhibited by the application of TEA or by increasing the external K^{+} concentration. In the whole cell clamp mode, application of ISO increased K^{+} outward current, and this effect was completely eliminated by propranolol. In the cell-attached patch, application of ISO or forskolin increased Ca^{2+}-activated K^{+} channel activity. Application of ISO to the bath in the outside-out patches or GTP in the inside-out patches stimulated Ca^{2+}-activated K^{+} channels. From the above results, both A-kinase dependent channel phosphorylation and direct GTP-binding protein mediated effect might be responsible for the the activation of Ca^{2+}-activated K^{+} channel by ISO in rabbit coronary smooth muscle cells. And this K^{+} channel activation also contributes to the ISO-induced vasorelaxation.

Key Words: Rabbit coronary smooth muscle cells, isoproterenol, Ca^{2+}-activated K^{+} channel, G-protein

The magnitude of coronary arterial tone, one of the important factors in the regulation of coronary blood flow, is mainly determined by the cytosolic Ca^{2+} concentration and Ca^{2+} sensitivity of the contractile element of coronary vascular smooth muscle cells (Conti and Adelstein, 1981; Kamm and Stull, 1985; Nelson et al. 1990). The concentration of intracellular Ca^{2+} ([Ca^{2+}]) is closely related to the magnitude of membrane potential of vascular smooth muscle cells which is controlled by hormones and drugs (van Breemen and Saldia, 1989; Nelson et al. 1990). When the membrane potential of smooth muscle cell is hyperpolarized, Ca^{2+} influx via sarcolemmal Ca^{2+} channel and Na^{+}/Ca^{2+} exchange decreased. These cause a decrease of [Ca^{2+}] and subsequent relaxation of smooth muscles (Mekata, 1986; Cook et al. 1988).

The large conductance Ca^{2+}-activated K^{+} channel play an important role in the regulation of membrane potential and vascular tone. A small increase in Ca^{2+}-activated K^{+} channel
activity can induces a profound hyperpolarization of membrane potential, because of the large number and high conductance of Ca\(^{2+}\)-activated K\(^+\) channels in smooth muscle cells (Bolton et al. 1985; Benham et al. 1986; Carl et al. 1990). Thus, an agent which changes the activity of the Ca\(^{2+}\)-activated K\(^+\) channel can affect the membrane potential and contractility of smooth muscle cells (Toro and Stefani, 1991). Beta adrenergic agonist, ISO, is widely used to relax smooth muscle cells. It has been shown that receptor stimulated by ISO causes vascular relaxation by hyperpolarization of membrane potential and the decrease of Ca\(^{2+}\) sensitivity (Ito et al. 1979; Conti and Adelstein, 1981; Honda et al. 1986). Augmentation of Ca\(^{2+}\)-activated K\(^+\) current by ISO is considered to be a key mechanism of ISO-induced vascular relaxation. One mechanism of Ca\(^{2+}\)-activated K\(^+\) channel regulation by ISO involves the indirect activation of the channel by adenylate cyclase-linked receptor stimulation leading to phosphorylation of the channel by cAMP-dependent protein kinase A (A-kinase) (Carl et al. 1991; Fan et al. 1993). However, it is not clear whether the activation of Ca\(^{2+}\)-activated K\(^+\) channel by ISO could be mainly or exclusively accounted for by the activation of adenylate cyclase.

In present paper, we measured the effect of ISO on the isometric tension to elucidate the contribution of Ca\(^{2+}\) sensitivity change during the vascular relaxation. We also investigated the mechanism of ISO-induced K\(^+\) channel activation under various patch clamp conditions.

MATERIALS AND METHODS

Preparation of coronary ring and measurement of tension

Albino rabbits (body wt. 2.0~2.5 kg) of either sex were anaesthetized by injecting 60 mg/kg sodium pentobarbital into the ear vein and they were exsanguinated by cutting a femoral artery. The left anterior descending coronary artery was extracted with neighboring cardiac muscle and then moved to a preparation chamber. The surrounding connective tissue was carefully removed with fine scissors and forceps under a surgical microscope. Coronary rings of 2~3 mm width were mounted using L-shape stainless hook in a perfusion chamber and its temperature were kept at 37°C.

Isometric tension of coronary rings were continuously recorded on polygraph using a Grass FT03 force displacement transducer. Before the experiment was begun tissue were equilibrated for 120 minutes under a 0.5 g of resting tension. The endothelium were removed by forcep and its functional integrity was checked by the method of Furchgott and Zawadzki (1980).

Preparation of coronary smooth muscle cells

The coronary artery strip was exposed longitudinally and bathed in Ca\(^{2+}\)/Mg\(^{2+}\) free Tyrode solution for 5 minutes. Additional 60 minutes bath was done after changing the solution containing 0.1% collagenase (Wako Co, Japan). The collagenase was washed out with same Tyrode solution and shaked with a fine glass rod. Coronary smooth muscle cells were resuspended in 1% albumin contained modified Tyrode solution (1 mM Mg\(^{2+}\), Ca\(^{2+}\) free Tyrode solution) and stored at 4°C for further experiment.

Measurement of channel currents

The activity of Ca\(^{2+}\)-activated K\(^+\) current was measured from various patch mode using a standard patch clamp techniques (Hamil et al. 1981). Patch clamp studies were performed with a commercial amplifier (Axopatch 1-C) and the data were stored on a computer hard disk and backed up with a tape back up system (Colorado Inc.). An AD/DA converter (Labmaster board, Axon Inc.) was employed for data acquisition (digitized at 5 KHz after filtering at 1 KHz) and for the generation of step voltage signals under control of IBM compatible computer using computer software (pClamp 5.5, Axon Inc.). Single channel conductance and open probability (NPo) of Ca\(^{2+}\) activated K\(^+\) channels were analysed using a pClamp 5.5 (Axon Inc, USA). Patch clamp experiment was taken under room tem-
Solution and drugs

Tyrode solution for cell preparation contains (mM): NaCl, 140; KCl, 5.4; CaCl₂, 1.8; MgCl₂, 1.0; HEPES, 10; Glucose 5.5; pH = 7.4 adjusted with Tris. The High K⁺ Tyrode solution used at tension development was made by equimolar replacing NaCl with KCl of Tyrode solution. The standard pipette-filling solution for whole cell and single channel recording was (in mM) KCl, 140; Glucose, 10; HEPES, 10; MgCl₂, 1; EGTA 1, (pH = 7.4). According to the experimental purpose, Tyrode solution or high K⁺ solution (KCl, 140; Glucose, 10; HEPES, 10; CaCl₂, 3.727; EGTA 5, pH = 7.4) was used as a perfusing solution. Pinacidil (gift from Dr. Kuriyama) was dissolved at 0.05 mM HCl solution, Isoproterenol was dissolved in 0.1mM ascorbic acid and forskolin was dissolved at 0.1% DMSO then diluted with the perfusate. All chemicals were purchased from Sigma Co.

RESULTS

Isoproterenol-induced vascular relaxation

Coronary rings were precontracted by replacing the bath solution with high K⁺-Tyrode solution and then the effect of ISO was measured. As shown in Fig. 1, application of ISO (1 μM) to the bath significantly relaxed the precontracted coronary rings elicited by low concentration of KCl (20 mM K⁺-Tyrode solutions) but had lesser effect on responses elicited by higher KCl concentration (80 mM K⁺-Tyrode solution). The percent change of relaxation by ISO during precontractions elicited by 20 mM and 80 mM external KCl was 74.6 ± 2.6% in 20 mM KCl solution and 31.2 ± 4.2% in 80 mM KCl solution.

Inhibition of ISO effect by high external KCl suggest the involvement of K⁺ channel opening in ISO-induced relaxation of coronary rings. To rule out this possibility, we measured the effect of K⁺ channel opener, pinacidil, at the same experimental conditions (Cook et al. 1988; Fan et al. 1990). Application of pinacidil (10 μM) showed similar results to the effect of ISO and the magnitude of relaxation by pinacidil was 98.7 ± 1.3% in 20 mM KCl solution and 13.6 ± 2.5% in 80 mM KCl so-
Activation of Ca\(^{2+}\)-activated K\(^{+}\) Channel by Isoproterenol

**Fig. 2.** Effects of TEA pretreatment on isoproterenol-induced relaxations.

A: Original recordings representing the effects of isoproterenol (1 µM) on high K\(^{+}\)-induced contractions and effects of TEA (1 mM) pretreatment on isoproterenol-induced relaxations. The inhibiting effect of TEA pretreatment on isoproterenol-induced relaxations were reduced at high external K\(^{+}\) conditions.

B: Isoproterenol-induced relaxation and the effect of TEA pretreatment were expressed as the percent of peak amplitude of precontraction. Data are means ± SE of values from 5 arterial rings. *: significantly greater inhibition of isoproterenol-induced relaxation by TEA in lower external K\(^{+}\)-conditions (20mM K\(^{+}\) vs 80mM K\(^{+}\) Tyrode solutions).

We further evaluated the involvement of K\(^{+}\) channel opening by pretreatment of tetraethylammonium chloride (TEA) during ISO-induced relaxations (Fig. 2). In the presence of TEA (1 mM), the relaxing effect of ISO on 20 mM KCl-induced contraction was significantly reduced (% change of relaxation: in the absence of TEA = 74.6 ± 2.6%, in the presence of TEA = 31.2 ± 4.2%, p < 0.01). However, in the case of 80 mM KCl-elicited contractions, pretreatment of TEA had a little effect on the relaxing effects of ISO (% change of relaxation: in the absence of TEA = 44.2 ± 3.5%, in the presence of TEA = 30.3 ± 4.0%, p > 0.1).

**Whole cell outward current change by ISO**

All experiments were performed with spindle shaped relaxed smooth muscle cells. After making a whole cell mode, depolarizing voltage pulses from a holding potential of −50 mV to +60 mV elicited an oscillating outward current which were increased along with the magnitude of depolarization. Application of 1 M ISO to the bath solution apparently increased the amplitude of the outward current through the entire duration and at all voltages. After removal of ISO, the activity of the outward current returned to the control level (Fig. 3A). To rule out the possibility whether this ISO-induced increase of outward current was due to the activation of beta receptor or not, propranolol was applied to the bath solution prior to ISO application. Fig. 3B shows the effect of a 1 µM ISO alone and combined application of ISO (1 µM) and propranolol (10 µM). The increase of outward current by ISO was completely blocked by propranolol pretreatment.

As reported in previous experiment (Ahn et al. 1994), the outward current in the coronary smooth muscle cell is due almost exclusively to opening of Ca\(^{2+}\)-activated K\(^{+}\) channels. To confirm this, we measured effect of membrane potential and TEA on K\(^{+}\) channel activity in inside-out and outside-out patch mode. After making an outside out patch, we changed the membrane potential from −20 mV to +30 mV. The open probability of K\(^{+}\) channel was increased along with the magnitude of depolarization (Fig. 4A). Application of
Fig. 3. Effects of propranolol pretreatment on isoproterenol-induced activation of K⁺ current.
A: Application of isoproterenol (1 μM) to the perfusate increased K⁺ outward currents in response to voltage steps of 300 ms spaced 10 mV from -30 mV to +60 mV was measured. The membrane potential was held to -50 mV and voltage pulses were applied at every 10s. Tyrode solution was used as a perfusing solution.
B: Effects of isoproterenol (1 μM) alone, and combined application of isoproterenol (1 μM) and propranolol (10 μM) on K⁺ outward current was measured. Membrane potential was clamped to -60 mV and changed to +50 mV for 300 ms at every 10s. Tyrode solution was used as a perfusing solution.

TEA (from 0.05 mM to 1 mM) to the bath in the outside-out patch significantly reduced the elementary current in a dose dependent manner (Fig. 4B) as reported by others (Langton...
Activation of Ca\textsuperscript{2+}-activated K\textsuperscript{+} Channel by Isoproterenol

Fig. 4. Membrane potential and external TEA-dependent change in Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel activity.

A: The membrane potential of the outside-out patch was changed from −20 mV to +30 mV and resulting single channel current activities were recorded. Channel activities were increased along with the depolarization of membrane potential. Tyrode solution was used as a perfusing solution.

B: The membrane potential of the outside-out patch was clamped to −10 mV and resulting single channel current activities were recorded (control). Concentration of TEA in the bath was increased stepwise from 0.05 mM to 1 mM. Single channel amplitudes were decreased along with the concentration of external TEA. Tyrode solution was used as a perfusing solution.

Changes of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel activity by ISO; Involvement of cAMP-dependent protein kinase pathway

After making a cell-attached patch, membrane potential was clamped to +60 mV and resulting Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel activity was recorded as a control. In this patch, multiple open state could be seen, especially after application of ISO to the bath, suggesting the presence of several active channels in this patch. The % increase of NPo after application of ISO to the bath was 236.3±64.3% (n=5). Activation of beta receptor by ISO might activate A kinase via activating adenylate cyclase, and the activation of A kinase may increase the activity of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels via channel phosphorylation (Carl et al. 1991; Fan et al. 1993). To confirm this possibility, we measured the effect of forskolin on this channel. Bath application of forskolin, which can activate adenylate cyclase, significantly increased the activity of Ca\textsuperscript{2+}-activated
Fig. 5. Effects of isoproterenol and Forskolin on Ca²⁺-activated K⁺ channel activity in cell-attached patch mode.
A: After making an giga seal, pipette potential was clamped to +60 mV and resulting single channel current activities were recorded (control). Application of isoproterenol (1 μM) to the perfusate caused a significant increase in Ca²⁺-activated K⁺ channel activities. Tyrode solution was used as a perfusing solution.
B: Application of forskolin (10 μM) to the perfusate caused a significant increase in Ca²⁺-activated K⁺ channel activities. Pipette potential was clamped to −10 mV. Tyrode solution was used as a perfusing solution.

K⁺ channel. After making an cell-attached patch mode, the membrane potential was held at −10 mV and the resulting channel activity was recorded as a control. The application of forskolin (10 μM) to the bath increased the NPo of Ca²⁺-activated K⁺ channel (Fig. 5B, 164.1±21.9%, n=2).

Direct modulation of Ca²⁺-activated K⁺ channel via G-protein

To examine the possible coupling between beta receptors and Ca²⁺-activated K⁺ channels, the channel activity was examined in an outside-out patches under physiologic concentration of Ca²⁺ (pCa 7) and GTP (0.1 mM) at the cytosolic surface. Addition of ISO (1 μM) to the bath inside-out patches stimulated channel activity (Fig. 6A). In three similar experiments, ISO increased the channel activity (% increase=193.2±27.3). The involvement of membrane delimited GTP-binding protein (G-protein) action on Ca²⁺-activated K⁺ channel was further evaluated by application of GTP to
Fig. 6. Effects of isoproterenol on Ca²⁺-activated K⁺ channel activity in cell-free excised patches.  
A: After making an outside-out patch, membrane potential was clamped to 0 mV and resulting single channel current activities were recorded (control). Application of isoproterenol (ISO, 1 μM) to the perfusate caused a significant increase in Ca²⁺-activated K⁺ channel activities. Tyrode solution was used as a perfusing solution and 0.1 mM GTP was added to standard pipette filling solution.  
B: After making an inside-out patch, membrane potential was clamped to 0 mV and resulting single channel current activities were recorded (control). Application of GTP (0.1 mM) to the perfusate caused a significant increase in Ca²⁺-activated K⁺ channel activities. Isoproterenol (1 μM) contained pipette solution was used. High K⁺ solution was used as a bath solution.
DISCUSSION

Involvement of $K^+$ channel activation in ISO-induced relaxation

Coronary arterial smooth muscle cells relax in response to circulating epinephrine, and it may be related to hyperpolarization or decrease of $Ca^{2+}$ sensitivity induced by ISO (Ito et al. 1979; Kamm and Stull, 1985; Shogakiuchi et al. 1991). In this study, we investigated whether hyperpolarization induced by ISO can modulate the contraction of smooth muscle cell or not. As shown in Fig. 1, higher external KCl (80 mM KCl) lessened the ISO-induced relaxation of coronary rings. This is the expected result if ISO relax the smooth muscle by inducing hyperpolarization. Because smooth muscle stimulated with 80 mM KCl is more depolarized than 20 mM KCl-stimulated smooth muscle and should be near the top of the L-type $Ca^{2+}$ channel current-voltage relationship (van Breemen et al. 1987). Therefore the alteration of $Ca^{2+}$ influx by the same membrane potential change was lesser in 80 mM KCl-stimulated smooth muscle cells than in the 20 mM KCl-stimulated one. Additionally, the driving force for $K^+$ is also reduced with higher $K^+$ because the electromotive force for $K^+$ ($E_m-E_K$) becomes smaller in 80 mM KCl than in 20 mM KCl. In this case, $K^+$ channel opening by some agonist would have less effect on membrane potential (Nelson et al. 1990; Chen and Rembold, 1992). Based on this analysis, our data best fit the hypothesis that ISO activate $K^+$ channels induce hyperpolarization and relaxation of smooth muscle. This hypothesis was further supported by the results of pinacidil and TEA. As shown in Fig. 1 and Fig. 2, the change of $K^+$ channel activity by pinacidil and TEA showed substantially the same results of ISO application as expected. These results suggest that ISO induces relaxation of smooth muscle by membrane hyperpolarization which is due to activation of $K^+$ channel. However, in tension measurements we can not directly demonstrate the type of $K^+$ channels which is activated by ISO and its underlying activating mechanisms.

Mechanism of ISO-Induced activation of $Ca^{2+}$-activated $K^+$ channel

$K^+$ channels observed in our experiments showed characteristic flickering block produced by external TEA (<1 mM) and the voltage dependent increase of open probability (Fig. 4). These findings are compatible with the $Ca^{2+}$-activated $K^+$ channel reported by others (Benham et al. 1986; Blatz and Magleby 1987; Yamaguchi et al. 1988; Hu et al. 1989; Langton et al. 1991). $Ca^{2+}$-activated $K^+$ channels are distributed over the various vascular smooth muscle cell membrane in high density and play an important role in determining the level of membrane potential and vascular contractility. So the agents which change the channel activity can greatly affect the vascular contractility (Carl et al. 1990; Kajoka et al. 1991; Brayden and Nelson 1992). The relaxing effect of beta adrenergic agonist are also believed to be associated with the activation of $Ca^{2+}$-activated $K^+$ channels (Kume et al. 1989; Kume et al. 1992; Fan et al. 1993). However, the underlying mechanism of channel activation by ISO is still controversial. One mechanism of $Ca^{2+}$-activated $K^+$ channel regulation by ISO involves the indirect activation of the channel by A-kinase dependent phosphorylation (Kume et al. 1989; Sadoshima et al. 1988; Reinhart et al. 1991; Fan et al. 1993). However, it is not clear whether the activation of $Ca^{2+}$-activated $K^+$ channel by ISO could be mainly or exclusively accounted for by the activation of adenylate cyclase or not. To rule out this possibility, we investigated the involvement of secondary messenger system on ISO-induced channel activation using a cell-attached patch. In the cell attached patch, the giga ohm seal between the patch pipette and the cell membrane forms a barrier against the lateral diffusion of these agents onto the patch. Thus the effects of the drug which was applied to the extrapatch bath solution on the channel can be attributed to actions of diffusible intracellular second messengers (Ogden and Stanfield, 1987; Fan et al. 1993). As shown in Fig. 5, the application of ISO (1 $\mu$M) and forskolin (10 $\mu$M) to the bath stimulated channel activity significantly. Our results suggest
that Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel are activated by A-kinase dependent channel phosphorylation.

The possibility that ISO can stimulate the Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels by a membrane-delimited action of activated G-protein was investigated using a cell-free excised patch system (Toro et al. 1990; Kume et al. 1992). As shown in Fig. 6, Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels were activated by physiological concentration of ISO (1 \mu M) in the outside-out patches (Fig. 6A) and by guanine nucleotides in inside-out patches (Fig. 6B). In the outside-out patches, we used the ATP free pipette solution to prevent the involvement of localized activation of A-kinase on channel activity and the application of ISO to the bath still stimulated channel activity independently of channel phosphorylation (Fig. 6A). Channel stimulation also readily demonstrated in the inside-out patches by using an exogenous GTP when ISO was present in the pipette solution. These results suggest that exogenous GTP or ISO can stimulate Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels through activating the endogenous G-proteins in the patch which does not require the A-kinase dependent channel phosphorylation. Thus, membrane delimited G-protein pathway plays an important role in the regulation of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (Yatani et al. 1987; Brown and Birnbaumer, 1988).

In summary, both A-kinase dependent channel phosphorylation and activated G-protein might be responsible for the the activation of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel by ISO in rabbit coronary smooth muscle cells. And this K\textsuperscript{+} channel activation also contributes to the ISO-induced vasorelaxation.

REFERENCES


Blatz AL, Magleby KL: Calcium-activated potassium channel. TINS 10: 463-467, 1987


Brayden JE, Nelson MT: Regulation of arterial tone by activation of Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels. Science 256: 532-535, 1992


Carl A, McHale NG, Publicover NG and Sanders KM: Participation of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels in electrical activity of canine gastric smooth muscle. J Physiol 423: 205-221, 1990

Chen XL, Rembold CM: Cyclic nucleotide-dependent regulation of Mn\textsuperscript{2+} influx, [Ca\textsuperscript{2+}], and arterial smooth muscle relaxation. Am J Physiol 263: C468-C473, 1992


Fan SF, Wang S, Kao CY: The transduction system in the isoproterenol activation of the Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel in guinea pig taenia coli myocytes. J Gen Physiol 102: 257-273, 1993


Honda K, Satake T, Takaki K, Tomita T: Effects
of relaxants on electrical and mechanical activities in the guinea-pig tracheal muscle. Br J Pharmacology 87: 665-671, 1986


