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The Alteration of Ca^{2+}-activated K^{+} Channels in Coronary Arterial Smooth Muscle Cells Isolated from Isoproterenol-induced Cardiac Hypertrophy in Rabbit

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It has been proposed that Ca^{2+}-activated K^{+} (K_{Ca}) channels play an essential role in vascular tone. The alterations of the properties of coronary K_{Ca} channels have not been studied as a possible mechanism for impaired coronary reserve in cardiac hypertrophy. The present studies were carried out to determine the properties of coronary K_{Ca} channels in normal and hypertrophied hearts. These channels were measured from rabbit coronary smooth muscle cells using a patch clamp technique. The main findings of the present study are as follows: (1) the unitary current amplitudes and the slope conductance of coronary K_{Ca} channels were decreased without changes of the channel kinetics in isoproterenol-induced cardiac hypertrophy; (2) the sensitivity of coronary K_{Ca} channels to the changes of intracellular concentration of Ca^{2+} was reduced in isoproterenol-induced cardiac hypertrophy. From above results, we suggest for the first time that the alteration of K_{Ca} channels are involved in impaired coronary reserve in isoproterenol-induced cardiac hypertrophy.

Key Words: Coronary Ca^{2+}-activated K^{+} channels, Isoproterenol-induced cardiac hypertrophy, Impaired coronary reserve, Patch clamp technique

INTRODUCTION

Cardiac hypertrophy is an initial adaptive response to several types of cardiovascular stress (Packer, 1992) and a major risk for development of heart failure and sudden cardiac death (Levy et al, 1990). Pressure or volume overload on the myocardium increases myocardial wall stress and hypertrophy may be seen as an attempt to normalize wall stress and oxygen demand (Kuhn, 1982; Chilian & Marcus, 1987). Although initially protective, the increased myocardial mass requires an increase in coronary blood flow to maintain function; indeed, cardiac hypertrophy may be associated with myocardial ischemia, even with angiographically normal coronary arteries (Pichard et al, 1981; Opherk et al, 1984). Reduced coronary reserve in response to physiological stress such as pacing (Vrobel et al, 1980; Bache et al, 1981; Bache, 1988), exercise (Murray et al, 1981; Hittinger et al, 1990), or the coronary vasodilator adenosine (Hittinger et al, 1989) is a recognized feature of cardiac hypertrophy. The reduced coronary reserve limits the ability of hypertrophied hearts to meet blood flow and metabolic requirements when demand is increased. Thus, despite normal myocardial oxygen consumption and myocardial perfusion per unit mass at rest, the hypertrophied heart is more vulnerable to ischemia (Malik et al, 1973). Elucidating the mechanisms of the impaired coronary reserve is therefore important to reduce the mortality from cardiovascular causes.

Many studies have shown the alteration of coronary circulation in cardiac hypertrophy and have suggested that several factors contribute to this phenomenon (Opherk et al, 1984; Dellsperger & Marcus, 1990). Studies of pressure/flow relations during maximal vasodilatation have demonstrated an increase in minimal coronary vascular resistance and an increased

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systolic flow impediment (O'Gorman et al, 1992; Radvan et al, 1995). Some studies have suggested a reduction in the number of resistance vessels in hypertrophied myocardium, others have suggested that arteriolar density normalize later in hypertrophy and there were no transmural differences in vascular morphology and no reduction in volume percentage capillary space (Cimini & Weiss, 1988; Bishop et al, 1993). From these results, structural alterations of coronary artery may not be the primary mechanism responsible for the reduced coronary reserve. Furthermore, in low and moderate grades of cardiac hypertrophy, an appropriate increase in coronary artery size has been reported (Villari et al, 1992).

Ca^{2+}-activated K^+ (K_{Ca}) channels are one of dominant ion channels found in the plasma membrane of vascular smooth muscle cells (Nelson & Quayle, 1995). The channels are known to play a major role in the regulation of smooth muscle excitability by control of resting membrane potential and termination of action potential (Benham et al, 1986). A growing body of evidence suggests that vasodilatation of coronary arteries is mediated by activation of K_{Ca} channels (Li et al, 1998; White et al, 2000). In fact, the hyperpolarization of vascular smooth muscle cells and subsequent vascular relaxation were elicited by opening of K_{Ca} channels (Holzmann et al, 1994; Node et al, 1998). We have hypothesized that the alteration of the properties of K_{Ca} channels plays an important role in impaired coronary reserve in the hypertrophied myocardium. However, the extent to which alterations of K_{Ca} channels occur in association with coronary arterial electrophysiology in cardiac hypertrophy has not been explored. The present studies were carried out to determine the properties of coronary K_{Ca} channels in normal and isoproterenol-induced hypertrophied hearts.

METHODS

Induction of cardiac hypertrophy

Cardiac hypertrophy was induced in New Zealand white rabbits by injection of isoproterenol (300 μg/kg) once daily for 10 days (Benjamin et al, 1989; Gillis et al, 1996). Age-matched control rabbits received the same amount of 0.9% NaCl solution only. The animals were used for experiments 24 h after the last injection. The degree of hypertrophy was estimated by measuring the blotted wet heart weight and the body weight and calculating the heart weight-to-body weight ratio.

Cell-isolation procedure

Single vascular myocytes were isolated from rabbit coronary arteries by enzymatic dissociation. Rabbits of either sex were anaesthetized with sodium pentobarbital (10 mg/kg I.V.). The heart was quickly removed and placed in a cold oxygenated Tyrodes solution composed of (in mM): 143 NaCl, 5.4 KCl, 5 N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), 0.33 NaH_2PO_4, 1 $MgCl_2$, 16.6 glucose, and 1.8 $CaCl_2$ (pH 7.4 with NaOH). The left anterior descending coronary artery was carefully removed with a portion of myocardium attached to it and was then pinned down to silicone elastomer layered in a dissecting Petri dish. The adhering ventricular myocardium and connective tissue were carefully removed under binocular examination. The arteries were then incubated for 30 min at room temperature in the same medium, except that calcium was omitted. Enzymatic dispersion was then initiated by incubating the artery for 30~40 min with collagenase (2.5 mg/ml, Wako Pure Chemical), bovine albumin (1 mg/ml, Sigma Chemical), and dithioerythritol (0.5 mg/ml) were added to a Ca-free Tyrodes solution. After 30~40 min of incubation with the enzymes, arteries were retrieved and rinsed several times with fresh Ca-free solution. Single smooth muscle cells were mechanically dispersed by triturating of the tissue pieces by using a Pasteur pipette in Kraft-Brühe (KB) solution composed of (in mM): 70 KOH, 50 *L*-glutamic acid, 50 KCl, 20 taurine, 20 KH_2PO_4, 3 $MgCl_2$, 20 glucose, 10 HEPES, and 0.5 ethylene glycol-bis (β-aminoethyl ether) *N, N, N', N'*-tetraacetic acid (EGTA) (pH 7.3 with KOH). When a sufficient number of relaxed spindle-shaped smooth muscle cells became apparent under microscopic examination, the isolated cells were cold-stored (4°C) until use.

Experimental procedure and electrophysiological techniques

All experiments were performed at room temperature. The recording chamber was mounted on a movable stage of an inverted microscope. The volume of the recording chamber was ~1 ml. Before each experiment, a sample of the stored supernatant containing

single cells was deposited in the experimental chamber, and the cells were allowed to settle for 20~30 min. They were then superfused for 5 min with normal Tyrodes solution before the patch-clamp experiment was started. Some of the cells contracted irreversibly during the initial perfusion. Patch-clamp experiments were only carried out on cells that remained in a relaxed state. Cell capacitance was measured by electrical compensation for capacity transients and series resistance was applied using the Axopatch 1D, from which cell capacitance was read directly. Single-channel currents were measured in inside-out patch configuration using a patch-clamp amplifier (Axopatch-1D, Axon Instruments, Foster City, CA, USA). Gigaohmseals were obtained using pipettes of 5~10 MΩ resistance pulled from borosilicate glass capillaries (Clark Electrochemical, Pangbourne, England) with a vertical puller (Narishige PP-83, Japan). Their tips were coated with Sylgard and fire polished. Patches were examined at a holding potential of 0~60 mV, depending on the level of control channel activity. After a patch was obtained, an equilibration period of ~5 min was allowed; patches that showed large fluctuations in channel activity over this period were discarded. Single-channel currents were digitized at a sampling rate of 48 kHz and stored in digitized format on digital audio tapes using a Biologic DTR-1200 recorder. For the analysis, the data were transferred to a computer (IBM-PC, 80486 DX2-66) with pCLAMP v 6.03 software (Axon Instruments, Burlingame, CA, USA) through an analogue-to-digital converter interface (Digidata-1200, Axon Instruments Inc.). Mean unitary currents were measured by averaging the digitized record, using cursors to select open periods. Amplitudes measured in this way were the same as those measured by fitting Gaussian curve to amplitude histograms. The open time histogram was formed from continuous recordings of more than 60 sec. The open probability (P_o) was calculated using the formula:

$$P_o = \left(\sum_{j=1}^{N} t_j j\right)/(T_d N)$$

Where t_j is the time spent at current levels corresponding to j=0, 1, 2, ... N channels in the open state, T_d is the duration of the recording and N is the number of channels active in the patch. The number of channels in a patch was estimated by dividing the maximum current that observed by the mean unitary current amplitude. P_o was calculated over 30-sec records.

Solutions and drugs

For inside-out patches, the bath solution was (in mM): 21.22 KOH, 123.78 KCl, 1 $MgCl_2$, 2.4 $CaCl_2$, 3 EGTA, 2 K-ATP, and 10 HEPES (pH 7.4 with KOH). The pipette solution contained (in mM): 145 KCl, 10 HEPES, 1 $MgCl_2$, 1 $CaCl_2$ (pH 7.4 with KOH). The Ca^{2+}, EGTA ratio was adjusted to give a pCa of 6.96. All chemicals and drugs were obtained from Sigma Chemical (St. Louis, MO, USA).

Statistical analysis

Data are presented as mean S.E. when appropriate. Unpaired Student's *t* test was used to assess the statistical significance of differences observed between control and hypertrophy. $P \leq 0.05$ was accepted as the level of significance.

RESULTS

Characteristics of the experimental model

Table 1 summarizes the characteristics of the rabbit cardiac hypertrophy model used in the present study. Heart weight and heart weight-to-body weight ratio were significantly greater in rabbits with isoproterenol-induced hypertrophy than in control (0.62±0.01 g/kg in control, n=14 and 0.70±0.01 g/kg in hypertrophy, n=26, $P<0.05$). With this in cardiac structure, the mean cell capacitance (C_m) was 24% greater for isolated coronary arterial myocytes from isoproterenol-

Table 1. Characteristics of rabbit isoproterenol-induced hypertrophy

	Control (n=14)	Hypertrophy (n=26)
Body weight, kg	1.29±0.06	1.36±0.05
Heart weight, g	7.93±0.41	9.66±0.33*
Heart wt/body wt, g/kg	0.62±0.01	0.70±0.01*
Heart rate	239.11±0.62	228±7.94

*P<0.05, compared to control

induced hypertrophy than for control coronary arterial myocytes ($P<0.05$; Fig. 1).

Comparing the properties of K_{Ca} channels in coronary arterial smooth muscle cells

Ca^{2+}-activated K^+ (K_{Ca}) channels are abundant in smooth muscle cells, and have been very well characterized in a variety of cell types (Carl et al, 1996). Furthermore, pharmacological (Ishikawa et al, 1993) and electrophysiological (Keef & Ross, 1987; Leblanc et al, 1994) properties of these channels have been studied in smooth muscle cells from the rabbit coronary artery.

The pipette and bath solutions contained 145 mM K^+ and 10^{-7} mM free Ca^{2+}. When inside-out patches were obtained, the coronary arterial smooth muscle cells from normal and hypertrophied heart showed a dominant large-conductance channel that were both Ca^{2+} and voltage dependent (see Fig. 2 and Fig. 4). Channel activity was seen at both positive and negative membrane potential in control cells. Initially we compared the single channel conductance of K_{Ca} channels in normal and hypertrophied cells. Fig. 2A shows a representative large-conductance unitary current from a normal cell and a hypertrophied cell

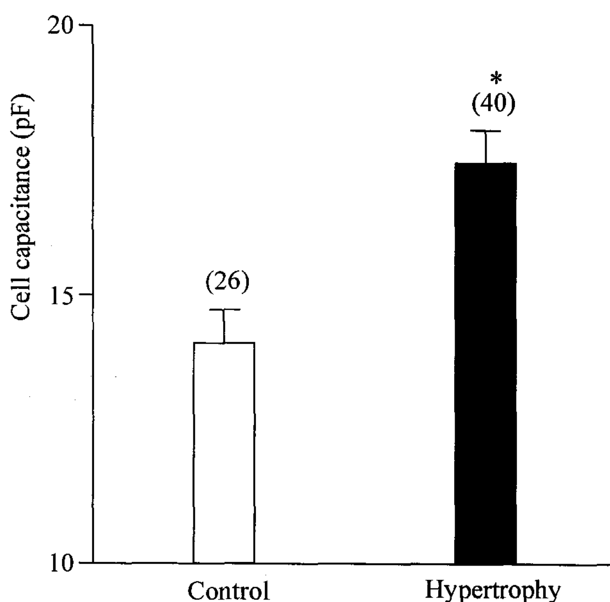

Fig. 1. Whole cell capacitance (C_m) of isolated coronary arterial myocytes from hypertrophic hearts (■) and control hearts (□). Numbers in parentheses represent number of myocytes. C_m values are means±S.E. *$P<0.05$ compared with control (unpaired *t*-test).

at positive membrane potential. The channels underlying these events appeared to be K^+ selective (data not shown) and have a single-channel conductance, measured as the slope of current-voltage relationship between 0 and +60 mV in symmetrical 145 mM K^+, of 321.13±2.00 pS (Fig. 2B, open circles, n=5). Fig. 2B shows the unitary current-voltage relations recorded at membrane potentials from 0 to +70 mV. Each single channel amplitude at various membrane potentials was reduced in hypertrophied cells in comparison with normal cells ($P<0.05$). A single-channel conductance in hypertrophied cells, measured as the slope of current-voltage relationship between +20 and +60 mV, was 265±2.55 pS (filled circles, n=6, $P<0.05$). The channel activity was voltage dependent in the positive voltage range. The NP_o in the hypertrophied cells was significantly less than that in normal cells between +30 and +50 mV (Fig. 2C; n=7 normal cells, 8 hypertrophied cells; $P<0.05$). To examine the gating kinetics of the channels, the open- and closed-time histograms were calculated at membrane potentials of +50 mV (Fig. 3). Most of the patches contained more than one functional K_{Ca} channels. In a few experiments, however, a single channel was recorded and open- and closed-times were successfully analyzed in normal and hypertrophied cells. The open-time histogram, which was analyzed from the current record filtered at a cutoff frequency of 10 kHz, revealed a single-exponential distribution with open-time constant (τ_o) of 55.73±3.25 ms in the control (n=3). In the hypertrophy, the open-time constant (τ_o =54.30±1.65 ms, n=3) did not differ from that in control ($P>0.05$). The closed-time histogram analysis using records filtered at a cutoff frequency of 10 kHz was fitted using a biexponential function, with constants of a fast (τ_{c1}) and a slow component (τ_{c2}). This analysis was performed with closed times >100 ms to be discarded. The value of τ_{c1} was not influenced by hypertrophy (τ_{c1}=0.71±0.04 ms in control, n=3 and τ_{c1}=0.72±0.05 ms in hypertrophy, n=3, $P>0.05$). The value of τ_{c2} was not also influenced by hypertrophy (τ_{c2}=76.42±43.58 ms in control, n=3 and τ_{c2}=44.85±18.50 ms in hypertrophy, n=3, $P>0.05$).

Ca^{2+} sensitivity of K_{Ca} channels in coronary arterial smooth muscle cells

We determined the difference of the Ca^{2+} sensitivity of K_{Ca} channels between normal and hypertrophied

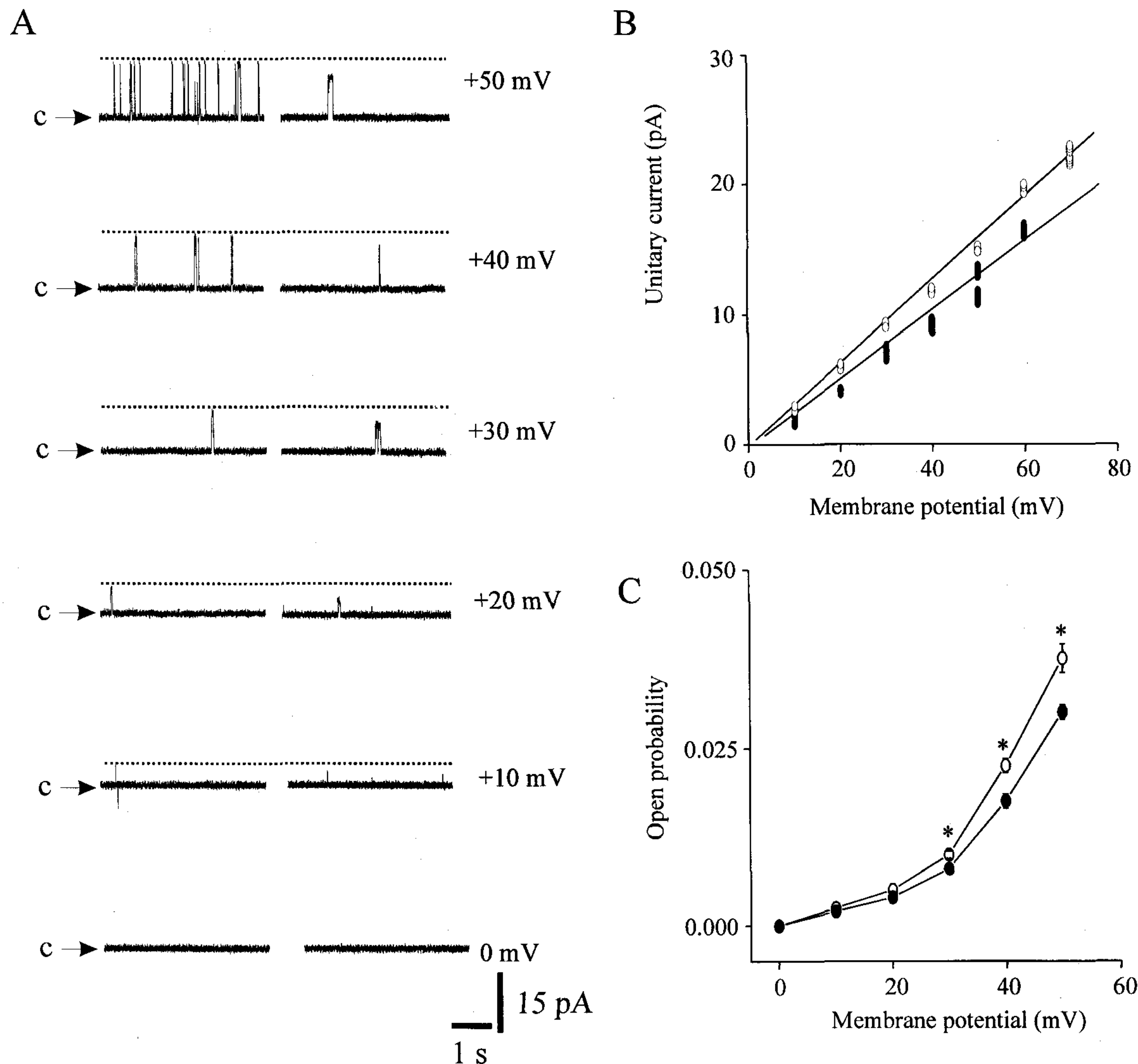

Fig. 2. A. K_{Ca} currents in coronary arterial smooth muscle cells in the inside-out patch configuration in normal and hypertrophied cells. B. Current-voltage (*I-V*) relationships of K_{Ca} channels in inside-out patches. Open symbols and dashed line are for control coronary myocytes, solid symbols and line for hypertrophied coronary myocytes. C. The NP_o of K_{Ca} channels increased with membrane potential in both normal (○; n=7) and hypertrophied (●; n=8) cells in the inside-out patch configuration. The NP_o in hypertrophied cells was less than that in normal cells between +30 and +50 mV. $^*P<0.05$ vs. normal cells.

cells. The activity of K_{Ca} channels, measured as NP_o, was correlated with the changes in intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$). The channel activity showed a very steep dependence on $[Ca^{2+}]_i$ and complete saturation beyond 0.07 μM. Fig. 4 shows the concentration-response relations (K_{Ca} channel activity vs $[Ca^{2+}]_i$) obtained from the two cell types. Each channel activity at six selected concentrations of Ca^{2+} was normalized to the channel activity recorded at 0.1 μM Ca^{2+} (maximum). At a low Ca^{2+} concentration of 0.03 μM, the channel activity is 94.9% of the maximum level in normal cells. However, at the same concentration of Ca^{2+}, the channel activity is only 67.8% of the maximum level in hypertrophied cells. The concentration-response relations fit well to the Hill equation. The half-maximum activation concentration (K_d) of Ca^{2+} was 14.88±0.43 nM in normal cells and 20.16±2.31 nM in hypertrophied cells (n=7, $P<0.05$) and the Hill coefficient is 4.14±0.27 in normal cells and 2.28±0.44 in hypertrophied cells (n=7, $P>0.05$). As shown in Fig. 4, the concentration-response curve of K_{Ca} channel to Ca^{2+} in

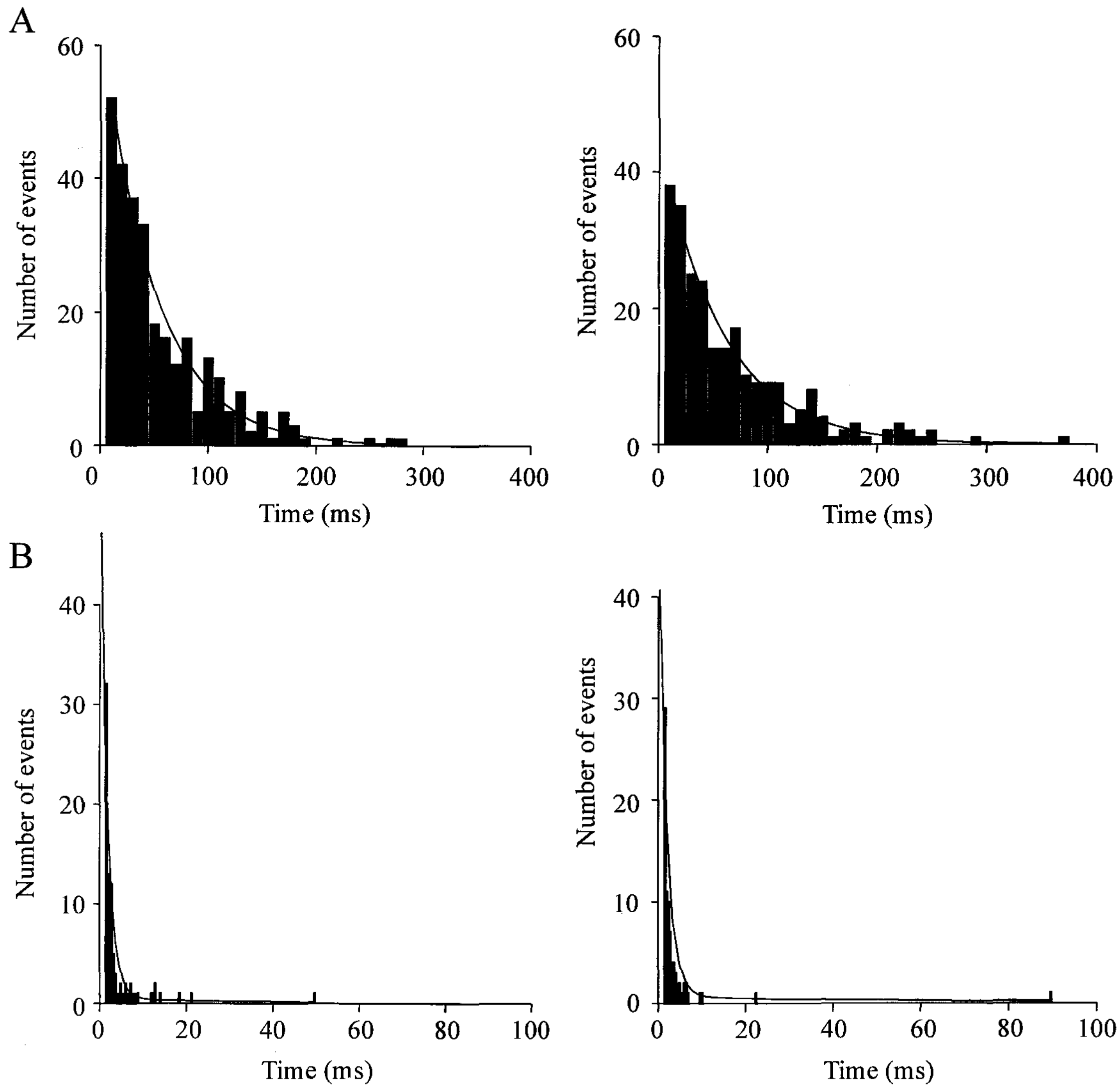

Fig. 3. Kinetic properties of K_{Ca} channels in hypertrophied (right panel) and normal (left panel) cells. Single-channel currents were recorded at +50 mV in inside-out patch configuration. Histograms of open (A) and closed (B) times within bursts were analyzed from current records filtered at cutoff frequency of 10 kHz. Smooth curves were fitted by single-exponential least squares method in the open-time and biexponential least squares method in the closed-time. Bin width is 0.3 ms. Each time constants were not influenced by hypertrophy ($P>0.05$, n=3). τ_o, Open-time constant; τ_{c1}, fast component of closed-time constant; τ_{c2}, slow component of closed-time constant, respectively.

hypertrophied cells is shifted to the right compared with that in normal cells. These data indicate that Ca^{2+} has a reduced stimulatory effect on K_{Ca} channels in hypertrophied cells.

DISCUSSION

To the best of our knowledge, this is the first study determining the features of coronary K_{Ca} channels in normal hearts and isoproterenol-induced hypertrophied hearts. We have demonstrated in the present study that cardiac hypertrophy was induced by daily administration of 300 μg/kg isoproterenol for 10 days. In this model, there is a 13% increase in heart weight-to-body weight (HW/BW) ratio. No change was seen in the lung weight to body weight ratio or in the liver weight to body weight ratio, excluding the presence of congestive heart failure in this model. On the basis of the 13% increase in HW/BW ratio, and the absence of congestive heart failure, this model can be classified as mild cardiac hypertrophy (Hart, 1994). Collins

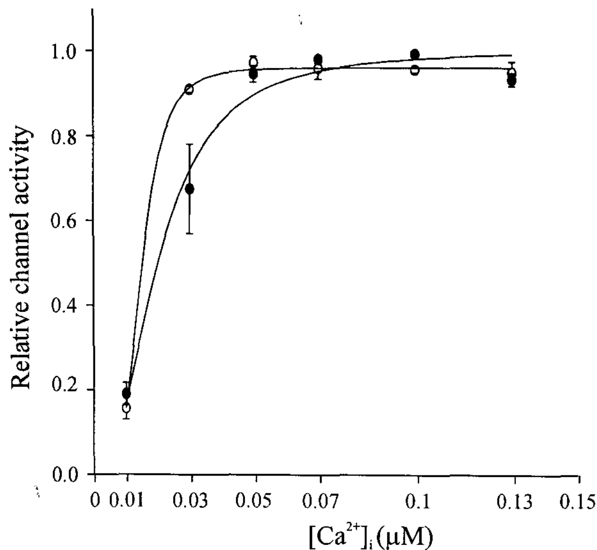

Fig. 4. Relationship between the activity of K_{Ca} channels and $[Ca^{2+}]_i$ in inside-out patches from coronary myocytes with hypertrophied (○) and normal (●) hearts. The channel activity for each Ca^{2+} concentration was normalized by referring to its value in 0.1 μM Ca^{2+}. The relationship between $[Ca^{2+}]_i$ and the normalized channel activity was fitted to Hill equation. Values are means S.E. for 7 cells. *Significantly different from control ($P<0.05$).

et al (1975) showed that repeated administration of a low dose of isoproterenol caused a 13% increase in both left and right ventricular weight in the rat and the degree of hypertrophy can be controlled by administering different doses or altering duration of administration. Thus this method provides a simple well-established model with low mortality and high reproducibility, which provides useful information that may have a relevant application to clinically observed disease (Mészáros et al, 1996). Moreover, significant changes in coronary cell occurred in our experimental models. The cell capacitance of coronary arterial myocytes was 24% greater in hypertrophy than in normal hearts, indicating that continuous infusion of isoproterenol may cause coronary myocyte hypertrophy as well as myocardial hypertrophy. Actually, isoproterenol acts direct stimulatory effects on protein synthesis in cardiac myocytes (Taylor & Tang, 1984; Morgan & Baker, 1991; Decker et al, 1993). But, RNA and protein syntheses of coronary arterial myocytes in cardiac hypertrophy have not been studied. In pressure-overloaded heart, total RNA content in the left anterior descending coronary artery and the left ventricular myocardium was increased, which indicates an early shift in protein synthesis in the left anterior descending coronary artery and the left ventricular myocardium (Gerova et al, 1996).

Many current experiments demonstrated that vasodilator responsiveness of coronary arteries to both exogenous and endogenous stimuli was reduced in cardiac hypertrophy (Bache et al, 1981; Murray & Vatner, 1981; Hittinger et al, 1990; Vassalli et al, 1995; Kingsbury et al, 2000). As a possible mechanism of reduced vasodilator responsiveness of coronary arteries, we hypothesized that coronary arterial smooth muscle vasoactivity was impaired by alteration of electrophysiological properties of coronary smooth muscle cell membrane. K_{Ca} channels have been proposed to play a substantial role in the regulation of membrane potential and hence the tone of vascular smooth muscle cells in arteries that display myogenic tone (Brayden & Nelson, 1992; Nelson et al, 1995; Nelson & Quayle, 1995). Actually, recent studies have shown that opening of K_{Ca} channels hyperpolarizes the cell membrane potential, leading to increase coronary artery blood flow (Node et al, 1998) or to produce vasorelaxation (Khan et al, 1998). On the other hand, Node et al (1996) have shown that iberiotoxin, charybdotoxin or TEA reduce coronary blood flow by inhibition of K_{Ca} channels.

The K_{Ca} channels are dependent on both voltage and Ca^{2+}. In the excised inside-out patch configuration, when free Ca^{2+} concentration on the inside membrane was the same for both normal and hypertrophied cells, the NP_o of these channels was still less in the hypertrophied cells than in the normal cells between +30 and +50 mV. This indicates that the decrease in NP_o in the hypertrophied cells may be caused partly by diminished voltage sensitivity of these channels. Moreover, in the present study, the unitary current amplitudes of K_{Ca} channels was reduced in the hypertrophied cells. We don't know the underlying mechanism of the reduced unitary current amplitudes of K_{Ca} channels. However, it is possible that the induction of coronary hypertrophy by repeated administration of isoproterenol may induce the alteration of protein synthesis for K_{Ca} channels. Although there are no studies available to show alteration of protein synthesis in coronary arteries, we suggest this possibility out of some studies in hypertrophied ventricles. Cardiac hypertrophy is known to induce gene switching to allow expression of various proteins (Izumo et al, 1988; Simpson et al, 1989; Lee et al, 1999) and interestingly, catecholamines induce protein synthesis

of some channels, whereas hypertrophy in some way causes also channel degradation (Mészáros et al, 1990). From above results, it is suggested that reduced the unitary current amplitudes and NP_o of K_{Ca} channels may lead to diminish whole-cell K_{Ca} currents, which affect the regulation function of K_{Ca} channels to myogenic tone.

To gain more information about the function of coronary K_{Ca} channels, we sought to examine the effects of enhancing its activity by elevating $[Ca^{2+}]_i$. Our results were consistent with the concept that elevation of $[Ca^{2+}]_i$, by opening of voltage-dependent Ca^{2+} channels or spontaneous Ca^{2+} release from sarcoplasmic reticulum stores, stimulated K_{Ca} channels (Hume & Leblanc, 1989; Ganitkevich & Isenberg, 1990) and showed that the channel activity was very steeply dependent on $[Ca^{2+}]_i$. But, responsiveness of K_{Ca} channels to $[Ca^{2+}]_i$ was reduced in coronary artery smooth muscle cells with isoproterenol-induced hypertrophy comparing to normal cells. The activation of K_{Ca} channels, caused by Ca^{2+} influx, is an important negative feedback mechanism that regulates the level of vascular tone (Nelson & Quayle, 1995). This regulatory pathway is likely to influence arterial tone in many vascular beds, including coronary circulation (Brayden & Nelson, 1992). Therefore, our results suggest that negative feedback action of K_{Ca} channels against elevation of $[Ca^{2+}]_i$ may be attenuated in hypertrophy, which could not well relax coronary artery in response to various stimuli. Alterations in K_{Ca} channel activity might also initiate or aggravate pathophysiological states such as vasospasm and ischemia (Brayden & Nelson, 1992).

In conclusion, these alterations of K_{Ca} channels may be involved in reduced coronary reserve in isoproterenol-induced cardiac hypertrophy. Therefore, to further determine the role that these alteration of K_{Ca} channels may change in coronary blood flow, experiments either in cannulated vessels or in wire-mounted vessels will be necessary. We will pursue these studies.

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