Vol. 193, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 28, 1993 Pages 240-247

Activation of ATP-Sensitive K^+ Channels by Cyclic AMP-dependent Protein Kinase in Cultured Smooth Muscle Cells of Porcine Coronary Artery

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Received April 15, 1993

SUMMARY The effects of cyclic AMP-dependent protein kinase on ATP-sensitive K^+ channels in cultured smooth muscle cells of the porcine coronary artery were investigated using the patch-clamp technique. Extracellular application of isoproterenol (1mM), a beta agonist, or forskolin ($2x10^{-5}$M), an activator of adenylate cyclase, activated these channels in cell-attached patch configurations, which were not blocked by phorbol 12-myristate 13-acetate (10^{-6}M), an activator of protein kinase C. Cyclic AMP-dependent protein kinase activated these channels in inside-out patch configurations. These results suggest that cyclic AMP-dependent phosphorylation modulates ATP-sensitive K^+ channels, in addition to its well known effects on Ca^{2+}-activated K^+ channels. The activation of ATP-sensitive K^+ channels by cyclic AMP-dependent phosphorylation contributes to hyperpolarization of the membrane and to the relaxation of vascular smooth muscle cells.

Adenosine 3',5'-cyclic monophosphate (cAMP) modulates ionic channels in various tissues as a second messenger (1, 2, 3). The K^+ channel plays an important role in controlling vascular tone in vascular smooth muscle cells. Ca^{2+}-activated K^+ channels (K_{Ca} channels) with large conductance are modulated by cAMP (4). In vascular smooth muscle cells, another K^+ channel, the ATP-sensitive K^+ channels (K_{ATP} channels), also modulates vascular tone

ABBREVIATIONS:
ATP; adenosine 5'-triphosphate, cAMP; 3',5'-cyclic monophosphate, ChTX; Charybdotoxin, DG; 1,2-diacylglycerol, EGTA; ethyleneglycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, IP_3; inositol 1,4,5-trisphosphate, K_{ATP} channels; ATP-sensitive K^+ channels, K_{Ca} channels; Ca^{2+}-activated K^+ channels, MOPS; 3-[N-morpholino] propanesulfonic acid, PKA; cAMP-dependent protein kinase, PKC; protein kinase C, PMA; phorbol 12-myristate 13-acetate, TEA; Tetraethylammonium ion.

0006-291X/93 $4.00

(5). cAMP in pancreatic β-cells activates K_{ATP} channels via cAMP-dependent protein kinase (PKA) (6). However, there have been no studies on the effects of cAMP-dependent phosphorylation on K_{ATP} channels in vascular smooth muscle cells. Accordingly, we examined the effects of PKA-mediated phosphorylation on K_{ATP} channels in cultured smooth muscle cells of the porcine coronary artery.

Materials and Methods

Single cell preparation

We cultured smooth muscle cells according to Ross's method (7). In brief, large epicardial coronary arteries of either side were excised from fresh porcine hearts obtained from a local slaughterhouse and cut into small pieces in normal Tyrode's solution after removing the endothelial tissue. The pieces were then explanted in culture dishes filled with medium 199 (Nissui Chemical, Hiroshima, Japan) containing 10 % fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.) and stored in a CO_2 incubator (5 % CO_2 at 37 °C). Four or five glass coverslips were placed on the bottom of each culture dish. For these experiments we used single smooth muscle cells that had migrated from the tissues during primary culture for 5-7 days and had adhered to the coverslips.

Solutions and chemicals

Tyrode's solution consisted of 137mM NaCl, 2.7mM KCl, 7.5mM Na-[3-[N-morpholino] propanesulfonic acid (MOPS)] buffer (pH 7.2), and 5.5mM glucose. High K^+ solution consisted of 140mM KCl (or K-aspartate) and 10mM K-MOPS buffer (pH 7.2). Ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)-Ca^{2+} buffer was used for adjusting Ca^{2+} concentrations of less than $5x10^{-6}$M. Normal Tyrode's solution contained 1.4mM $CaCl_2$. ATP, cAMP, PKA, catalytic subunit of PKA, isoproterenol, forskolin and phorbor 12-myristate 13-acetate (PMA) were obtained from Sigma (St.Louis,U.S.A.). Tetraethylammonium ion (TEA) was obtained from Wako Chemical Co., Osaka, Japan. Charybdotoxin (ChTX) was obtained from Peptide Institute Incorp., Osaka, Japan.

Electrophysiological measurements

Membrane currents were recorded in cell-attached and inside-out configurations with a patch-clamp amplifier (model EPC-7, List Medical Electronics, Darmstadt, FRG), as described by Hamil et al. (8). Soft glass patch pipettes prepared by a electrode puller (PP-83, Narishige Scientific Institute Laboratory, Japan) were used after Sylgard coating. The electrical resistance of the patch pipettes was 5-7 MΩ for single-channel recording. Experiments were conducted at a temperature of 35-37 °C. As cAMP had previously been reported to activate K_{Ca} channels (4), our objective was to clarify the effects of cAMP on K_{ATP} channels. K_{Ca} channel activities were much larger in conductance than those of K_{ATP} channel. Therefore, concentrations of 1mM TEA or of 10^{-7}M ChTX, which specifically block the K_{Ca} channels (9), were used to avoid the effects of K_{Ca} channel activities. Data were stored in a PCM recorder (model PCM-501ES, Sony Co., Tokyo, Japan) with a low pass filter (3kHz). The software used to analyze the data on single-channel currents was kindly provided by Dr.Y.Kurachi, Tokyo University. When there were multiple channels in the patch, the open probability was considered as "one minus the closed-channel fraction."

Results

In a preliminary study, we found that K_{ATP} channel activities were infrequent in the smooth muscle cells of the porcine coronary artery when the Ca^{2+} concentration in the pipette was 10^{-6}M in cell-attached patch configurations (5). Figure 1A shows the effects of isoproterenol, a beta

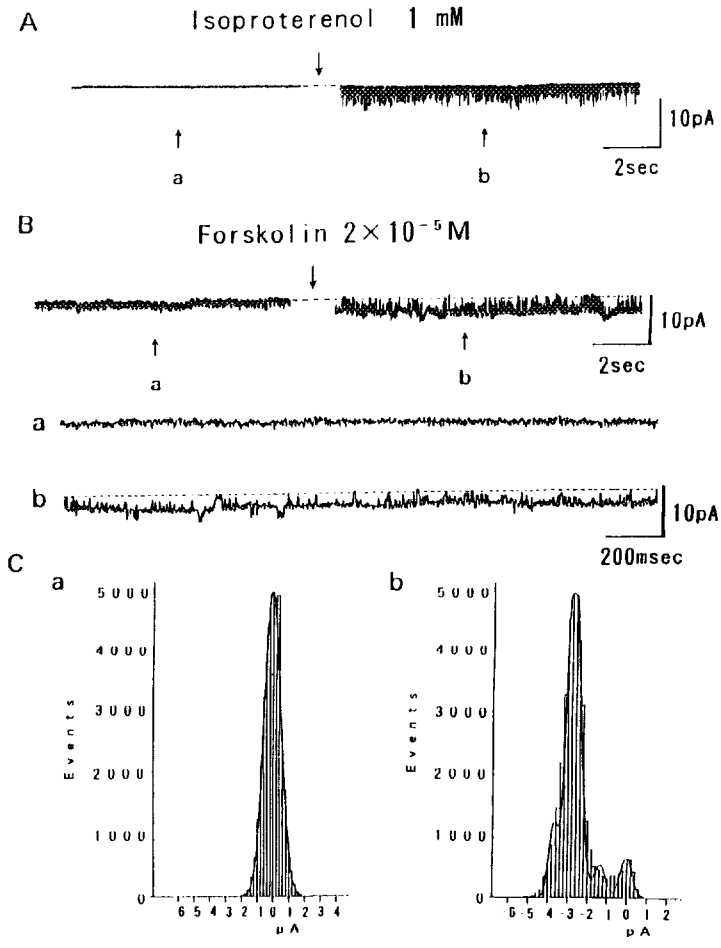

Figure 1. (A) Augmentation of K_{ATP} channels by isoproterenol in cell-attached patch configurations. Single channel currents were recorded in cell-attached patch configurations (tracing a) at a pipette voltage (Vp) of 60mV, i.e., patch membrane potential (Vm) is about -60mV. The bath solution contained 140mM KCl, 10mM K-MOPS, and 2mM Ca^{2+}, and the pipette solution contained 140mM KCl, 10mM K-MOPS, 10^{-6}M Ca^{2+}, and 1mM TEA. The K_{ATP} channels were activated by extracellular application of 1mM isoproterenol (tracing b). Downward recordings indicate the inward-directed transmembrane currents, and the dashed lines show the zero current level in this and other Figures. (B) Augmentation of K_{ATP} channels by forskolin in cell-attached patch configurations. Single channel currents were recorded in cell-attached patch configurations (tracing a) at a pipette voltage (Vp) of 30 mV, i.e., patch membrane potential (Vm) is about -30mV. The bath solution contained 100mM K-aspartate, 40mM KCl, 10mM K-MOPS, and 2mM Ca^{2+}, and the pipette solution contained 140mM KCl, 10mM K-MOPS, 10^{-6}M Ca^{2+}, and 1mM TEA. The K_{ATP} channels were activated by extracellular application of $2x10^{-5}$M forskolin (tracing b). (C) Amplitude histograms obtained from the results in Figs 1B-a and b.

agonist, on K_{ATP} channels in cell-attached configurations. Single channel activities were observed infrequently in the control (tracing a). Application of isoproterenol (1mM) to the bath solution enhanced the activities of channels (tracing b) (n=4). In inside-out patch configurations, these channels were blocked by ATP and glibenclamide, and were thought to be K_{ATP} channels (data not shown).

We studied the effects of forskolin, an activator of adenylate cyclase, on K_{ATP} channels in cell-attached configurations (Figure 1B). Application of forskolin ($2x10^{-5}$M) to the bath solution activated K_{ATP} channels. The open probability was 0.045 $\pm$ 0.074 (mean $\pm$ S.E., n=5) before the application of forskolin, and 0.517 $\pm$ 0.078 after the application of forskolin. Similarily application of dibutyryl cAMP (1mM) activated K_{ATP} channels in cell-attached configurations (data not shown). Application of phosphodiesterase inhibitors such as amrinon (10^{-4}M) or 3-isobutyl-1-methylxanthine (10^{-4}M) augmented the effects of forskolin on K_{ATP} channels (data not shown). These channels had a conductance of 30 pS in symmetrical 150 mM K^+ solution, and none of these agents did not alter unitary conductance.

We also studied the effects of PMA, an activator of protein kinase C (PKC), on K_{ATP} channels in cell-attached configurations (Figure 2). Application of PMA (10^{-6}M) to the bath solution did not significantly alter the open probability of K_{ATP} channels (n=3). We further tested high concentration of PMA (10^{-5}M), but PMA did not block K_{ATP} channels. In addition, K_{ATP} channels were not blocked by 1-oleoyl-2-acetylglycerol ($3x10^{-5}$M), an activator of PKC (data not shown).

Figure 3 shows the effects of PKA in inside-out patch configurations. In inside-out configurations, K^+ channels with a conductance of 30pS were recorded (tracing a). These channels were suppressed by applying 1mM ATP to the cytosolic side (tracing b). Activity of these channels was not changed by applying 10^{-4}M cAMP alone to the cytosolic side (tracing c). Application of PKA (20 units/ml) to the cytosolic side activated these K^+ channels (tracing d). Similar activations were observed with the catalytic subunit of PKA

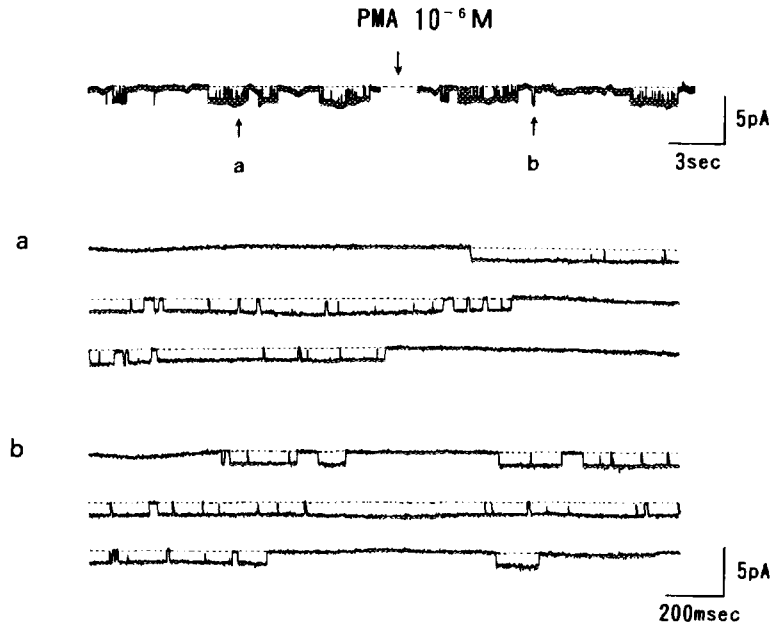

Figure 2. Effects of PMA on K_{ATP} channels in cell-attached patch configurations. Single channel currents were recorded in cell-attached patch configurations (tracing a) at a pipette voltage (Vp) of 40mV. The bath solution contained normal Tyrode's solution, and the pipette solution contained 140mM KCl, 10mM K-MOPS, 10^{-7}M Ca^{2+}, and 1mM TEA. K_{ATP} channels were not blocked by extracellular application of 10^{-6} M PMA (tracing b).

without cAMP. The open probability was 0.061 ± 0.076 (mean ± S.E., n=5) before the application of PKA, and 0.530 ± 0.167 in the presence of both cAMP and PKA. These results indicate that K_{ATP} channels are activated through cAMP-dependent phosphorylation. We also studied the effects of cytosolic Ca^{2+} on cAMP-PKA-induced K_{ATP} channel activation. Addition of 5mM EGTA to the bath solution (cytosolic side) slightly, but not significantly, suppressed the K_{ATP} channels (tracing e) (0.307 ± 0.133).

Discussion

cAMP is a second messenger in stimulating the β-receptor and contributes to a variety of cell responses (10). Ionic channels are among the targets of second messengers such as cAMP and cGMP in various tissues, and are modulated by these messengers (1, 2, 3, 11). The numerous K^+ channels in smooth muscle cells can be separated in various ways. Two types of K^+ channels, the K_{Ca} channels and K_{ATP} channels, have mainly been observed in cell-attached and

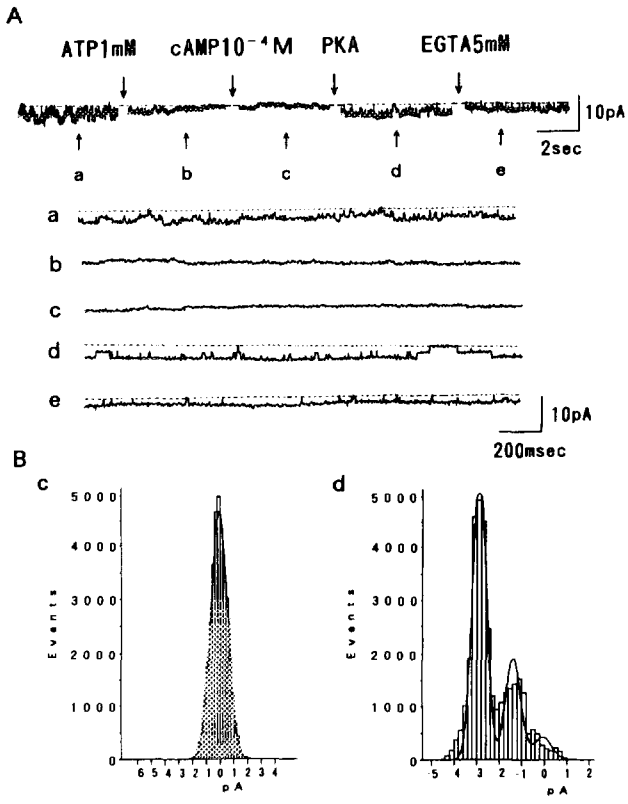

Figure 3. (A) Augmentation of K_{ATP} channels by PKA in inside-out patch configurations. Single channel currents were recorded in inside-out patch configurations (tracing a) at a pipette voltage (Vp) of 50mV, i.e., patch membrane potential (Vm) is -50mV. The bath solution contained 140mM KCl, 10mM K-MOPS, and 10^{-7}M Ca^{2+}, and the pipette solution contained 140mM KCl, 10mM K-MOPS, 10^{-6}M Ca^{2+}, and 10^{-7}M ChTX. By application of 1mM ATP to cytosolic side, K_{ATP} channels were almost blocked (tracing b). Channel currents were not changed by applying 10^{-4}M cAMP to the cytosolic side (tracing c). K_{ATP} channels were activated by applying PKA (20units/ml) to the cytosolic side (tracing d). Addition of 5mM EGTA to the cytosolic side did not significantly suppress K_{ATP} channels (tracing e). (B) Amplitude histograms obtained from results in Figs 3A-c and d.

inside-out patch configurations in cultured smooth muscle cells from the porcine coronary artery (5, 12). Sadoshima et al. (4) also demonstrated that cAMP modulated Ca^{2+}-activated K^+ channel in cultured smooth muscle cells of rat aortas. However, the effects of PKA and PKC on K_{ATP} channels have not been reported in smooth muscle cells.

Beta-agonists and forskolin activate adenylate cyclase and increase cAMP in the cell. In the present study, the extracellular application of isoproterenol or forskolin activated the K_{ATP} channels in cell-attached configurations with a pipette containing TEA or ChTX. In addition, K_{ATP}

channels were activated by cytosolic application of PKA in excised inside-out patch configurations, indicating that this activation was mediated via cAMP-dependent phosphorylation of the channel. However, this activation did not require Ca^{2+}, which differs from the case of the K_{Ca} channels.

PKC is reported to produce a sustained contraction of smooth muscle cells, and endothelin, angiotensin II and vasopressin in the pipette, which activate PKC through production of 1,2-diacylglycerol (DG), are reported to block K_{ATP} channels in cell-attached configurations (5, 12, 13). While PKC would be expected to block K_{ATP} channels, we found that PMA, an activator of PKC, did not block those channels. These results indicate that the blockade of K_{ATP} channels by these vasoactive substances is not due to the production of DG. Thus, the blockade of K_{ATP} channels might be due to other substances e.g. inositol 1,4,5-trisphosphate (IP_3) or direct modulation of K_{ATP} channels by these vasoactive substances.

In our previous studies as well as those of others, K_{ATP} channels were activated by K^+ channel openers such as cromakalim and nicorandil (5). Few studies have shown that activation of the K_{ATP} channels is controlled by physiological substances. The K^+ permeability is one of the most important factors that controls the membrane potential. Thus, the opening of these channels result in hyperpolarization and vasodilation. Among the many K^+ channels in vascular smooth muscle cells, only the K_{ATP} channels are highly active in physiological conditions and control the resting membrane potential. K_{Ca} channels are activated during depolarization and/or increase the cytosolic Ca^{2+} concentration and increase the outward current, which counteracts contraction by hyperpolarizing membrane. These findings indicate that cAMP not only counteracts contraction by opening the K_{Ca} channels but also controls the resting membrane potential and resting vascular tone by opening K_{ATP} channels.

Acknowledgments

We thank Professor Susumu Ito of the Second Department of Internal Medicine, School of Medicine, the University of Tokushima, for his guidance and encouragement. This study was supported by the Cell Science Research

Foundation and by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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