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Direct inhibition of a PKA inhibitor, H-89 on K_V channels in rabbit coronary arterial smooth muscle cells

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Abstract

We examined the effects of the protein kinase A (PKA) inhibitor H-89 on voltage-dependent K^+ (K_V) currents in freshly isolated rabbit coronary arterial smooth muscle cells, using a whole-cell patch clamp technique. H-89 inhibited the K_V current in a concentration-dependent manner, with a K_d value of 1.02 μM. However, the PKA inhibitors KT 5720 and Rp-8-CPT-cAMPS did not significantly alter the K_V current or the inhibitory effects of H-89 on the K_V current. Moreover, H-85, a structurally similar but inactive analog of H-89, showed similar inhibitory effects on the K_V channel. H-89 had no effect on the voltage-dependency of activation or inactivation, or on recovery kinetics. These results suggest that in rabbit coronary arterial smooth muscle cells, H-89 inhibits the K_V current directly by blocking the pore cavity, an effect independent of PKA inhibition.

Keywords: Protein kinase A; H-89; Voltage-dependent K^+ currents; Coronary arterial smooth muscle cells

In vascular smooth muscle cells, several subtypes of K^+ channels have been identified, including Ca^{2+}-activated K^+ (BK_{Ca}) [1], ATP-sensitive K^+ (K_{ATP}) [2,3], inward rectifying K^+ (K_{IR}) [4], and voltage-dependent K^+ (K_V) [5–7] channels. Among these, the K_V currents are a major contributor to the regulation of membrane potential [1]. Activation of K_V channels by membrane depolarization in response to pressure or to vasoconstrictors may limit that depolarization [8]. Indeed, inhibition of K_V channels by 4-aminopyridine depolarizes smooth muscle membranes and constricts many arteries [9].

The cAMP-dependent protein kinase (PKA) phosphorylates the hydroxyl group of serine or threonine residues on a number of protein substrates, including ion channels. In vascular tissues, PKA is fundamentally important, involved in both immediate regulation of effector functions and in the control of cellular growth [10,11]. K^+ channels are important targets of PKA-mediated signaling pathways. PKA has been reported to activate BK_{Ca}, K_{ATP}, and K_{IR} currents in coronary arterial smooth muscle cells [12–15]. While there have been relatively few reports of K_V channel modulation by vasodilators in systemic vascular smooth muscle, β-adrenoceptor stimulation activated K_V currents via PKA in rabbit portal vein [16], and it has been suggested that activation of K_V channels by prostacyclin may contribute to endothelium-dependent relaxation in rabbit cerebral arteries [17].

To investigate the role of PKA in various cell types, including those of vascular smooth muscle, pharmacological inhibitors have been widely used in both in vivo and in vitro studies. Among the PKA inhibitors, H-89 has been reported to have a useful specificity for PKA, with little

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inhibitory effect on the activity of protein kinase G (PKG) or of other protein kinases [18]. However, its utility in intact cells can be limited by side effects due to its action on other proteins. For example, H-89 directly blocks $K_V1.3$ channels expressed in Chinese hamster ovary (CHO) cells, rat epithelial Na^+ channels, and the sarcoplasmic reticulum Ca^{2+}-ATPase isolated from ferret ventricular myocytes; these effects are independent of its inhibition of PKA activity [19–21]. In the present study, we investigated the effect of H-89 on K_V channels of freshly isolated coronary arterial smooth muscle cells from rabbit and demonstrated that it inhibits K_V currents in a PKA-independent manner.

Methods

Cell isolation. This study was conducted in accordance with the guiding principles for the care and use of animals by the Seoul National University Animal Use and Care Committee. Single smooth muscle cells were isolated from rabbit coronary arteries as described previously [22]. Briefly, New Zealand White rabbits (1.8–2.3 kg) of either sex were simultaneously anesthetized by ketamine (35 mg kg^{-1}) and xylazine (5 mg kg^{-1}), injected with heparin (100 U kg^{-1}) through the ear vein. The coronary arteries were isolated and cleaned of connective tissue in the normal Tyrode solution under a stereomicroscope. The arteries were transferred to normal Tyrode solution without $CaCl_2$ (Ca^{2+}-free solution) for 10 min and then incubated in the Ca^{2+}-free solution containing papain (1 mg ml^{-1}) for 25 min. After the remaining enzyme was cleaned in the Ca^{2+}-free solution, the arteries were incubated in the Ca^{2+}-free solution containing collagenase (2.8 mg ml^{-1}) for 20 min. Single smooth muscle cells were obtained by gentle trituration with a Pasteur pipette in Kraft–Brühe (KB) solution, stored at 4 °C, and used on the day of preparation.

Solutions and chemicals. The normal Tyrode solution contained (mM) 143 NaCl, 5.4 KCl, 0.33 NaH_2PO_4, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 5.0 Hepes, and 16.6 glucose, adjusted with NaOH to pH 7.4. The KB solution contained (mM) 70 KOH, 50 L-glutamate, 55 KCl, 20 taurine, 20 KH_2PO_4, 3 $MgCl_2$, 0.5 EGTA, 10 Hepes, and 20 glucose, adjusted with KOH to pH 7.3. The pipette solution contained (mM) 115 K-aspartate, 25 KCl, 5 NaCl, 1 $MgCl_2$, 4 Mg-ATP, 10 BAPTA, and 10 Hepes, adjusted with KOH to pH 7.25. To block K_{ATP} currents and Ca^{2+}-activated currents, we included 4 mM Mg-ATP and 10 mM BAPTA in the pipette solution. In all experiments, the bath solution contained iberiotoxin (100 nM) to block the BK_{Ca} channel. All pharmacological compounds were dissolved in distilled water or in dimethyl sulfoxide (DMSO) to make stock solutions, which were diluted in the normal Tyrode solution to the concentration used. The concentration of DMSO in the final solution was less than 0.1%, which had no effect on the current recording. KT 5720 was purchased from Tocris Cookson (Ellisville, MO, USA). Rp-8-CPT-cAMPS was purchased from Biologic Life Science Institute (Bremen, Germany). H-85 was purchased from Seikagaku Kogyo (Tokyo, Japan). H-89 and iberiotoxin were purchased from Sigma Chemical (St. Louis, MO, USA).

Electrophysiology and data analysis. The whole-cell currents were recorded from single smooth muscle cells using patch clamp technique. An Axonpatch-1C amplifier (Axon Instruments) and digital interface (NI-DAQ 7, National Instruments) coupled to an IBM-compatible computer were used for patch clamp and data acquisitions. All experimental parameters were controlled using Patchpro software developed by our group. The patch pipettes were pulled from borosilicate capillaries (Clark Electromedical Instruments, Pangbourne, UK) using a puller (pp-830, Narsige, Japan). We used patch pipettes with a resistance of 2–3 MΩ, when filled with the pipette solutions. Liquid junction potentials between the bath and pipette solution were offset and then the GΩ seal was achieved by suction. Data acquisition was performed at a sampling rate of 1–2 kHz and filtered at 0.5 kHz.

Origin 6.0 software (Microcal Software, Northampton, MA) was used for data analysis. Data are presented as means ± SE of the mean. Student's t test was used for the test of significance ($P < .05$).

Results

H-89 inhibition of the K_V current in a dose-dependent manner

Fig. 1 illustrates the effects of H-89 on K_V currents in rabbit coronary arterial smooth muscle cells. Under control conditions (Fig. 1A), the K_V current rose rapidly to a peak and then showed slow inactivation during a depolarizing pulse in steps of 20 mV from −80 to +60 mV, starting at a holding potential of −60 mV. Tail currents were recorded upon repolarization to −40 mV. The K_V current decreased in the presence of 1 μM H-89 (Fig. 1B). A steady-state was reached within 1 min of applying a solution containing 1 μM H-89. The washout of the drug partially restored the current to below 60% of the control level (data not shown). The current–voltage (I–V) relationship showed that the magnitude of the K_V current was lowered to a similar extent by H-89 at the peak and at steady-state. Fig. 1C summarizes the I–V relationship in the steady-state.

Fig. 2 shows the concentration dependence of the effects of H-89 on K_V currents. Examples of inhibition of K_V current by 0.03, 0.1, 0.3, 1, 3, 10, and 30 μM H-89 are presented for cells exposed to a +40 mV depolarizing pulse from a holding potential of −60 mV in Fig. 2A. The inhibition by H-89 was again similar for both the peak and the steady-state K_V currents. For the steady-state inhibition, a nonlinear least-squares fit of the Hill equation to the concentration–response data at +40 mV yielded a K_d value of 1.02 ± 0.11 μM and a Hill coefficient of 1.21 ± 0.12 (Fig. 2B).

Effects of H-89 on activation and steady-state inactivation of K_V currents

The voltage dependency of activation and inactivation of K_V currents was evaluated. Activation curves were obtained from a double-pulse protocol starting from a holding potential of −60 mV. Channels were activated by the application of short depolarizing pulses to potentials between −60 and +50 mV in steps of 10 mV. The activating pulse in the first step of this protocol was 100 ms, as determined from the activation time course of Fig. 1. The second pulse stepped the membrane potential to −40 mV, and the peak value of the tail current was measured. The normalized current amplitude was considered to be an estimate of channel activation, and the data were fitted with a Boltzmann function. Fig. 3A shows the voltage dependence of activation under control conditions and in the presence of 1 μM H-89. H-89 produced no significant shift in the activation curve for the K_V current. The potential of the half-maximal activation ($V_{1/2}$) and the slope value (k) were

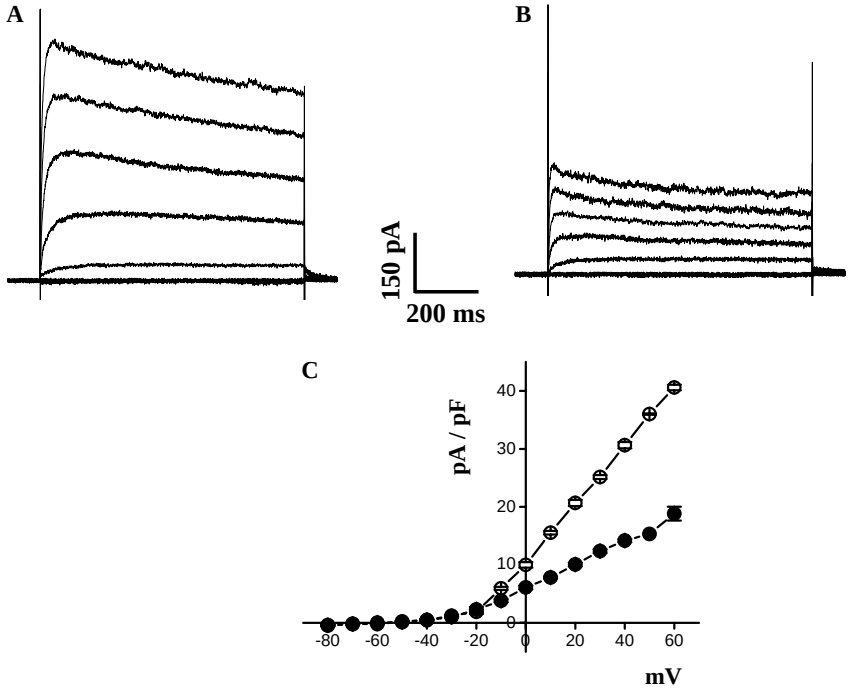

Fig. 1. H-89 inhibits K_V currents. K_V current response to 20 mV step pulses between −80 and +60 mV from a holding potential of −60 mV (A) under control conditions and (B) with application of 1 μM H-89. (C) Steady-state current–voltage relationship. At the end of the step pulses in steps of 10 mV, current amplitudes were measured and plotted against voltage under control conditions (○) and in the presence of 1 μM H-89 (●) ($n = 5$).

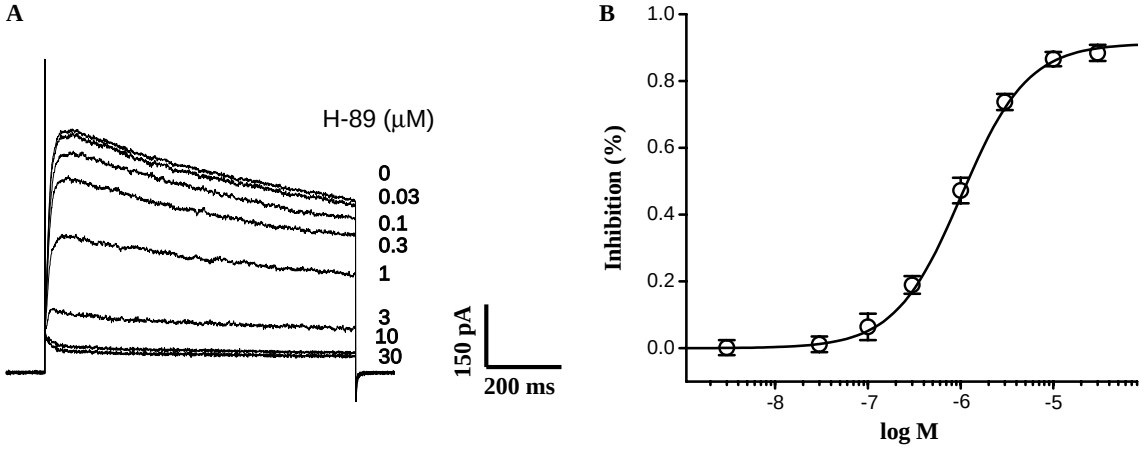

Fig. 2. H-89 inhibits K_V currents in a dose-dependent manner. (A) Representative current traces recorded by applying a depolarizing pulse of +40 mV from a holding potential of −60 mV. Each trace was recorded in the presence of 0, 0.03, 0.1, 0.3, 1, 3, 10, or 30 μM H-89. (B) Summarizing the data by averaging the percent inhibition of K_V currents at the end of the depolarizing pulse of +40 mV (○) and fitting to the Hill equation, represented as a smooth line ($n = 4$).

-13.12 ± 0.39 mV and 9.17 ± 0.32 under control conditions, and -14.40 ± 0.52 mV and 8.99 ± 0.52 in the presence of 1 μM H-89, respectively.

Steady-state inactivation curves were obtained using another double-pulse protocol. Currents were activated by a test step to +40 mV, after a 10-s conditioning pre-pulse at each voltage. The steady-state current amplitude in the test pulse was normalized to the peak amplitude after a pre-pulse potential, and the data were fitted with another Boltzmann function. Again, H-89 showed no significant effect on the inactivation curve of the K_V current. The potential of the half-maximal inactivation ($V_{1/2}$) and slope value (k) were -34.59 ± 1.04 mV and 9.47 ± 0.74 under control conditions, and -33.95 ± 0.42 mV and 10.17 ± 0.37 in the presence of 1 μM H-89, respectively (Fig. 3B).

Effect of H-89 on recovery kinetics

We investigated recovery kinetics using a double-pulse protocol as illustrated in the inset to Fig. 4. The interpulse interval was varied from 10 ms to 10 s. Fig. 4 shows the

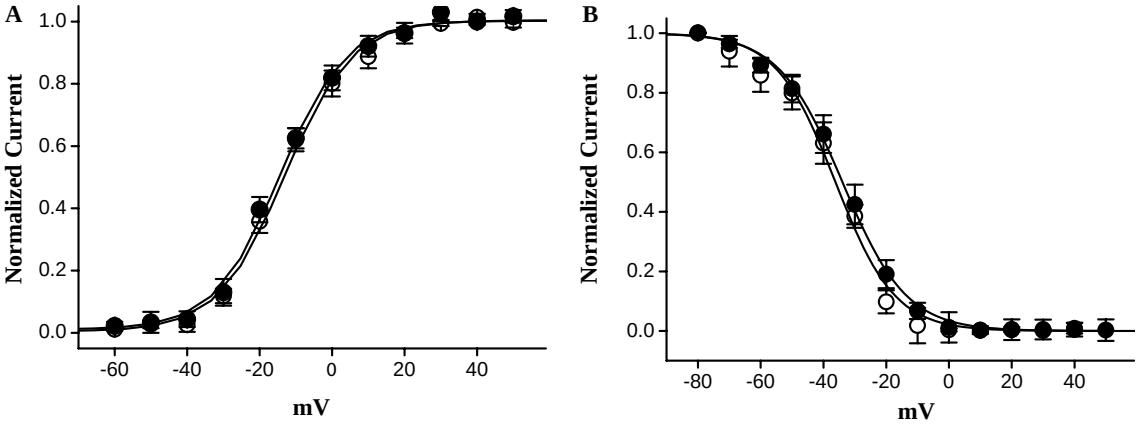

Fig. 3. Voltage dependence of activation and inactivation of the K_V current. (A) Activation curve obtained using a double-pulse protocol under control conditions (○) and in the presence of 1 μM H-89 (●). Data were fitted with a Boltzmann equation, represented as a smooth line ($n = 4$). (B) Steady-state inactivation curve obtained from using another double-pulse protocol under control conditions (○) and in the presence of 1 μM H-89 (●). Data were fitted with another Boltzmann equation, represented as a smooth line ($n = 4$).

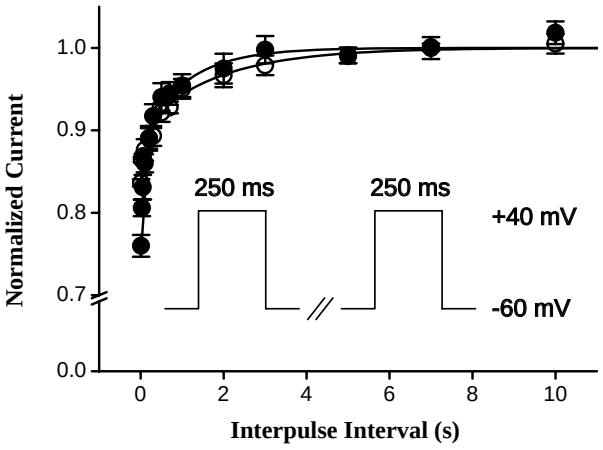

Fig. 4. Analysis of the recovery kinetics of the K_V current. To measure the degree of recovery, a double-pulse protocol was applied, as described in the inset. The amplitude of the current elicited by the second test pulses was normalized to that of the first test pulse under control conditions (○) or in the presence of 1 μM H-89 (●). Data were fitted to a single exponential function, represented as a smooth line ($n = 4$).

steady-state current amplitudes normalized to the first pulse of the double-pulse protocol. The data were fitted to a single exponential function with recovery time constants of 1.03 ± 0.12 s under the control conditions and 1.11 ± 0.26 s in the presence of 1 μM H-89, respectively. These data show that H-89 had no significant effect on recovery time constants.

Effects of other protein kinase A inhibitors on K_V channels

To investigate whether the inhibition of the K_V current by H-89 was indeed PKA-independent, we first tested the effect of other PKA inhibitors on the K_V channel. Rp-8-CPT-cAMPS and KT 5720 are commonly used PKA inhibitors that are structurally different from H-89. Figs. 5A and B show the effects of KT 5720 on K_V currents. Unlike H-89, KT 5720 failed to inhibit K_V currents. Furthermore, KT 5720 had no significant effect on the inhibition of K_V current by 1 μM H-89. At +40 mV, the K_V current densities were 30.67 ± 0.47 pA/pF under control conditions, 29.95 ± 0.57 pA/pF in the presence of 1 μM KT 5720, and 15.54 ± 0.83 pA/pF in the presence of 1 μM KT 5720 and 1 μM H-89, respectively. Similar results were obtained with the application of Rp-8-CPT-cAMPS (Figs. 5C and D). At +40 mV, the current densities were 29.13 ± 0.93 pA/pF under control conditions, 29.36 ± 0.72 pA/pF in the presence of 10 μM Rp-8-CPT-cAMPS, and 15.62 ± 0.77 pA/pF in the presence of 10 μM Rp-8-CPT-cAMPS and 1 μM H-89, respectively.

Fig. 5E shows the effect of 1 μM H-85, which despite having a similar structure to H-89 does not inhibit PKA. H-85 also decreased K_V currents, and to a similar extent as H-89 (Fig. 5F; 32.41 ± 0.90 pA/pF under control conditions, 16.93 ± 0.39 pA/pF in the presence of 1 μM H-85, and 16.24 ± 0.87 pA/pF in the presence of 1 μM H-89). These results suggest that the inhibitory effect of H-89 on K_V currents is direct, rather than via PKA inhibition.

Discussion

For several reasons, the decrease in K_V current seems to be attributable to direct causes and not to inhibitory influences on PKA. A previous report suggested that K_V currents were not affected by PKA inhibitors [23], and this study found that unlike other PKA inhibitors, H-89 decreased K_V currents. Furthermore, H-85, which has a structure similar to that of H-89 and is considered an inactive form, had similar effects on K_V current. Inhibition by H-89 occurred more rapidly (within 1 min) than would be expected if the effects were caused by PKA inhibition. This short exposure time for reaching steady-state inhibition cannot be easily explained by the inhibition of PKA activity. Two other PKA inhibitors, Rp-8-CPT-cAMPS and KT 5720, which are structurally different from H-89, had no

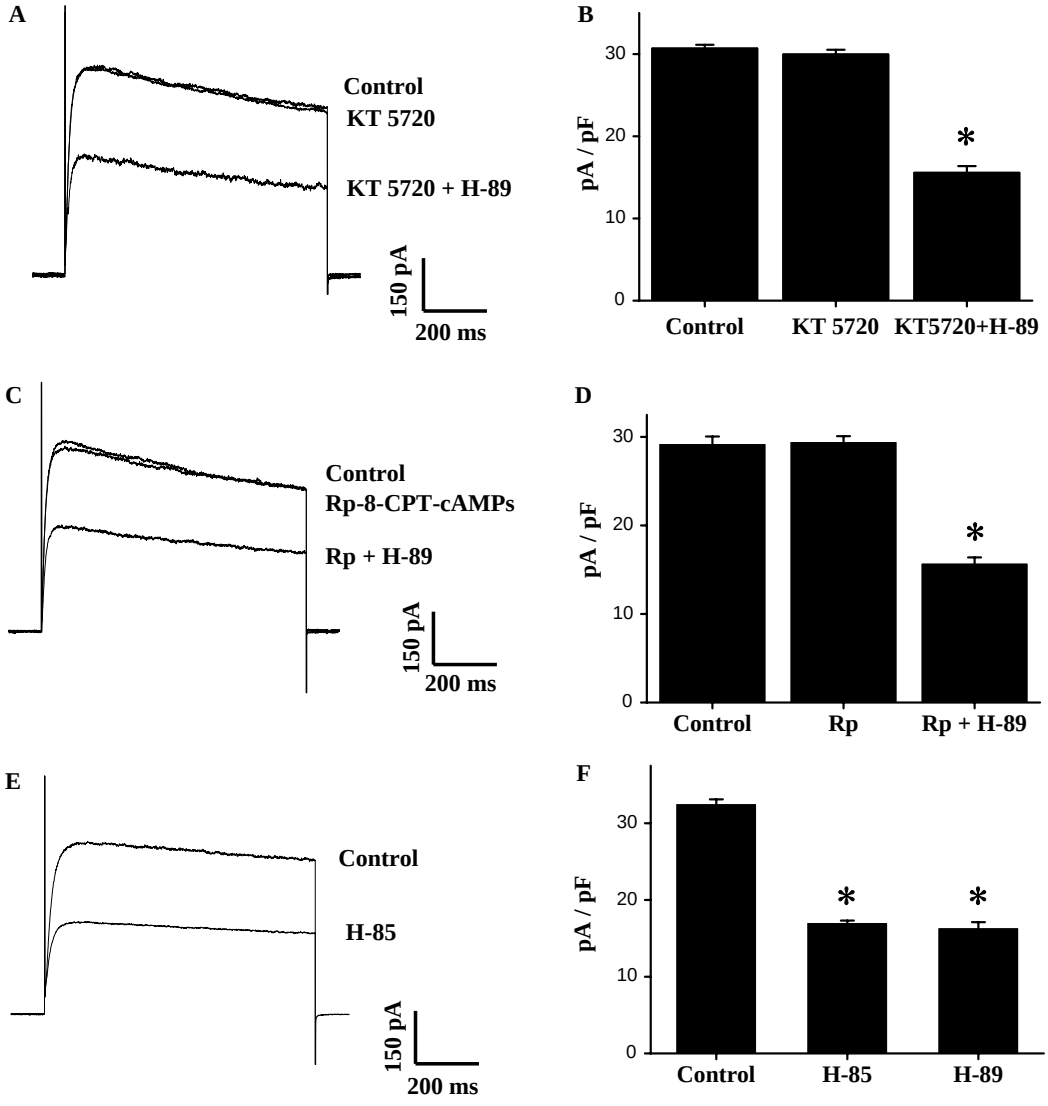

Fig. 5. Effects of other PKA inhibitors and H-85. (A) Representative traces obtained under control conditions in the presence of 1 μM KT 5720 and in the presence of both 1 μM KT 5720 and 1 μM H-89, applying a depolarizing pulse to +40 mV from a holding potential of −60 mV. (B) Summarized data from traces in (A) ($n = 4$). (C) Application of the PKA inhibitor Rp-8-CPT-cAMPS (Rp, 10 μM) using the same protocol as for KT 5720. (D) Summary of the data obtained after application of Rp or H-89 ($n = 4$). (E) Representative traces obtained in the absence or presence of 1 μM H-85 using the same pulse protocol as in (A). (F) Summary of the current density under control conditions in the presence of 1 μM H-89 and in the presence of 1 μM H-85 ($n = 5$). *$P < 0.01$.

effect on K_V channels, nor did they modify the inhibition of K_V currents by H-89. These results strongly suggest that H-89 directly interacts with K_V channels and inhibits the current independently of PKA effects.

Several subtypes of K^+ channel have been identified and their roles have been characterized in vascular smooth muscle cells of the coronary artery, including BK_{Ca} [1], K_{ATP} [2,3], K_{IR} [4], and K_V [5–7] channels. Of these, the K_V channel is one of the major components regulating the membrane potential [1,24]. K_V channels modulate crucial effector systems that mediate agonist-induced changes in vascular tone by altering membrane potentials in the coronary arteries [25,26]. In the present study, we have shown that K_V channels are affected by H-89 and H-85. This may have important implications for the study of PKA regulation in coronary vascular function.

In the previous studies, Choi et al. [19] reported that H-89 inhibited $K_V1.3$ channels stably expressed in Chinese hamster ovary cells. From their results, which showed acceleration of channel inactivation, they suggested that the inhibition by H-89 is due to an open-channel block. In the present study, however, inhibition of K_V currents by H-89 was not associated with accelerated inactivation during the application of depolarizing pulses. Furthermore, to investigate whether the inhibitory effect of H-89 on K_V channel is due to an open block, we tested both the kinetics of recovery from H-89-induced inhibition and use-dependency. As shown in Fig. 4, recovery kinetics showed no significant change. Use-dependency tests with pulses of 1 or 2 Hz (data not shown) also showed no effect of 1 μM H-89. The differences between the studies might be due to recording from different types of channels, the K_V 1.3

channel expressed in CHO cells and the K_V channel of freshly isolated smooth muscle cells. Furthermore, H-89 did not seem to modulate the voltage-sensitivity of the channel based on no significant alternation on the activation and the inactivation curves as shown in Fig. 3. In these rabbit coronary arterial cells, we suggest that the inhibition of K_V channels of freshly isolated smooth muscle cells by H-89 is due to the direct blocking of the pore cavity.

The H-series chemicals, which act as protein kinase inhibitors, have 5-isoquinolinsulfonamide as a key functional group. This functional group sits in the position normally occupied by a purine in the active site of protein kinases. The amino acids exposed in the pore cavity of some ion channels are similar to those in the active site of PKA and could interact via hydrogen bonds or van der Waals forces with the H-series compounds [18,27]. In PKA active sites, Val-123 is involved in hydrogen bonding to these compounds, and Leu-49, Val-57, Ala-70, Lys-72, and Leu-173 participate in van der Waals interactions with H-89 [28]. The sequence analysis of K_V 1.1, 1.2, 1.3, 1.5, and 3.1 channels identifies a number of amino acids that are candidates for interaction with H-series compounds: Glu-161, Ala-166, and Ala-170 prior to the S1 segment; Ala-242 between the S2 and S3 segments; Leu-312, Leu-315, Leu-319, Ala-321, Glu-325, Leu-326, Leu-328, and Leu-330 between the S4 and S5 segments; Val-373 in the selective filter of the pore; Ala-401, Leu-402, Val-404, Val-406, Val-408, and Glu-418 C-terminal to the S6 segment, near the pore cavity (amino acid position numbers used are based on K_V 1.1). Such interactions may be responsible for the inhibitory effects of H-89 and H-85.

A previously reported K_i of H-89 for PKA inhibition was 0.048 ± 0.0008 μM in an in vitro system; the IC_{50} was 135 nM, and the maximum effective concentration was 1 μM [18,29,30]. The concentration of H-89 usually employed to inhibit the effects of PKA ranges from 1 to 50 μM. For this reason, we used 1 μM H-89 in this study to explore inhibitory effects on K_V current and calculated a K_d for this effect of H-89 to be 1.02 ± 0.11 μM. Taking into consideration these differing IC_{50}s, the maximum effective concentration for PKA inhibition, and the range of concentrations in general use, the application of 1 μM H-89 appears likely to have major pharmacological consequences. Therefore, care is required when designing and evaluating studies that use H-89 as a PKA inhibitor in the investigation of ion channels and vascular function.

Acknowledgments

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