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The protein kinase A inhibitor, H-89, directly inhibits K_{ATP} and Kir channels in rabbit coronary arterial smooth muscle cells

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Abstract

The effects of the protein kinase A (PKA) inhibitor H-89 on ATP-sensitive K^+ (K_{ATP}) and inward rectifier K^+ (Kir) currents were examined in rabbit coronary arterial smooth muscle cells using the patch clamp technique. The H-89, in a dose-dependent manner, inhibited K_{ATP} and Kir currents with apparent K_d values of 1.19 ± 0.18 and 3.78 ± 0.37 μM, respectively. H-85, which is considered as an inactive form of H-89, inhibited K_{ATP} and Kir currents, similar to the result of H-89. K_{ATP} and Kir currents were not affected by either Rp-8-CPT-cAMPs, which is a membrane-permeable selective PKA inhibitor, or KT 5720, which is also known as a PKA inhibitor. Also, these two drugs did not significantly alter the effects of H-89 on the K_{ATP} and Kir currents. These results suggest that H-89 directly inhibits the K_{ATP} and Kir currents of rabbit coronary arterial smooth muscle cells independently of PKA inhibition.

Keywords: H-89; ATP-sensitive K^+ channel; Inward rectifier K^+ channel; Protein kinase A; Coronary artery

Protein kinase A (PKA) is an enzyme that phosphorylates serine/threonine residues of various protein substrates. The K^+ channels are important targets of PKA-mediated signal pathways. For example, the activation of PKA has been reported to activate Ca^{2+}-activated K^+ (BK_{Ca}), ATP-sensitive K^+ (K_{ATP}), and inward rectifier K^+ (Kir) currents in coronary arterial smooth muscle cells [1–4]. To investigate the role of PKA in K^+ channel activation, 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 on the PKA inhibition with little effect on the activity of protein kinase G (PKG) and other protein kinases [5]. However, its usefulness in intact cells can be limited by unexpected actions on other targets. For example, H-89 has been shown to directly block Kv1.3 channels expressed in Chinese hamster ovary cells, Na^+ channels in rat epithelium, and sacroplasmic reticulum Ca^{2+}-ATPase isolated from ferret ventricular myocytes [6–8].

Considering the significance of PKA in vascular smooth muscle function, information about the side actions of the PKA inhibitor, H-89, on vascular system is necessary to correctly interpret experimental results using this agent. In the present study, we demonstrate that H-89 directly inhibits K_{ATP} channels, which play an important role in controlling vascular tone [3] and Kir channels, which are expressed in small diameter vessels (<100 μm) and contribute to resting tone in coronary arterial smooth muscle cells (CASMCs) of rabbit [4,9] at concentrations similar to those that inhibit PKA.

Methods

Cell preparation. Single smooth muscle cells were isolated enzymatically from rabbit coronary artery. New Zealand White rabbits (~2.0 kg)

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of either sex were anesthetized with sodium pentobarbitone (50 mg kg^{-1}) and were injected simultaneously with heparin (100 U kg^{-1}) into the ear vein. The hearts were removed and immersed in ice-cold normal Tyrode solution. The left anterior descending coronary arteries were dissected. Before the enzymatic treatment of the coronary arteries (see below), the diameter of each dissected artery was measured using a video edge detector (Crescent Electronics, Sandy, UT, USA). The arteries were incubated at 37 °C in nominally Ca^{2+}-free normal Tyrode solution for 15 min. Each artery was cut open along the longitudinal axes and the outer and inner surfaces were cleaned of the adventitia and endothelium, respectively. Thereafter, single cells were obtained using enzymatic digestion of the arteries as follows. The arteries were incubated for 20–25 min in Ca^{2+}-free normal Tyrode solution containing (in mg/ml) 1.0 papain, 1.5 bovine serum albumin (BSA), and 1.5 dithiothreitol (DTT). The coronary artery was then further digested for 15–20 min in Ca^{2+}-free normal Tyrode solution containing (in mg/ml) 2.8 collagenase, 1.5 BSA, and 1.5 DTT. Following the aforementioned enzyme treatment, each artery was rinsed with Kraft–Brühe (KB) solution. Single smooth muscle cells were obtained by gentle trituration with a fire-polished Pasture pipette, stored at 4 °C, and used on the day of preparation.

Solution. Normal Tyrode solution contained (in mM): NaCl, 140; KCl, 5.4; NaH_2PO_4, 0.33; $CaCl_2$, 1.8; $MgCl_2$, 0.5; Hepes, 5; glucose, 16.6; adjusted to pH 7.4 with NaOH. The recordings were made under conditions of symmetrical 140 mM KCl using an extracellular solution of the same composition as that listed above, except that the concentration of KCl was 140 mM and NaCl was omitted. KB solution contained (in mM): KOH, 70; L-glutamate, 50; KH_2PO_4, 20; KCl, 55; taurine, 20; $MgCl_2$, 3; glucose, 20; Hepes, 10; EGTA, 0.5; adjusted to pH 7.3 with KOH. To record the K_{ATP} currents, we used a low ATP pipette solution containing (mM): K-aspartate, 115; KCl, 25; NaCl, 5; $MgCl_2$, 5; Mg-ATP, 0.1; EGTA, 0.1; Hepes, 10; with the pH adjusted to 7.25 using KOH. To record the Kir currents, the concentration of Mg-ATP was increased to 4 mM (to minimize the activity of K_{ATP} channels), and the concentration of $MgCl_2$ was lowered to 1 mM.

Drugs. All pharmacological compounds were prepared as stock solution in water or dimethylsulfoxide (DMSO) at >1000 times the concentration used during experiment. H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide), pinacidil, and glibenclamide were purchased from Sigma (St. Louis, MO, USA). 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). The final concentration of DMSO in the bath solution was always <0.1%, and we confirmed that this concentration of DMSO did not affect the currents that were recorded.

Electrophysiology. The membrane currents were recorded in the whole-cell configuration, using Axopatch 1C amplifier (Axon instruments, Union, CA). All experiment parameters, such as pulse generation and data acquisition, were controlled using the PatchPro software, developed by our group. Recording electrodes were pulled from thin-walled borosilicate capillaries (Clark Electromedical Instruments, Pangbourne, UK) using a PP-83 vertical puller (Narishige, Tokyo, Japan). The voltage and current signals were filtered at 0.5–1.0 kHz and were sampled at a rate of 1–3 kHz. We used patch pipettes with a resistance of 3–4 MΩ when filled with the above pipette solution. The liquid-junction potentials between normal Tyrode and pipette solution, which were calculated based on ionic mobilities, were −3 mV. Since they are not large and liquid-junction potential between the pipette solutions and intracellular solutions was not known, we did not make correction for junction potentials in presenting and analyzing data.

Statistics. Origin 6.0 software (Microcal Software, Northampton, MA) was used for data analysis. Interaction kinetics between drugs and channels was described on the basis of a first-order blocking scheme. The apparent affinity constant (K_d) and Hill coefficient (n) were obtained by fitting concentration-dependent data to the following Hill equation:

$$f = 1/\{1 + (K_d/[D])^n\}$$

in which f is the fractional inhibition ($f = 1 - I_{drug}/I_{control}$) at the test potential and [D] represents different drug concentrations.

Data are presented as means ± SEM. Statistical analyses were performed by Student's t test. A value of $P < 0.05$ was defined as statistically significant.

Results

H-89 inhibits K_{ATP} channels in rabbit coronary arterial smooth muscle cells

We investigated the effect of H-89 on the K_{ATP} channels in coronary arterial smooth muscle cells (CASMCs). To record the K_{ATP} currents, extracellular K^+ was increased to 140 mM, and whole cell recordings were made at a holding potential of −60 mV to minimize the activity of voltage-dependent K^+ channels. To increase the size of the K_{ATP} currents, we used the K_{ATP} channel opener pinacidil (10 μM) and an intracellular solution with a low ATP concentration (0.1 mM).

Fig. 1A shows that the application of 3 μM (left panel) and 10 μM (right panel) H-89 inhibited the K_{ATP} current in CASMCs (56.87 ± 6.34% inhibition at 3 μM, 89.46 ± 3.37 at 10 μM, respectively). Increasing the concentration of H-89 increased the level of inhibition of the K_{ATP} current. The concentration-dependence of the K_{ATP} current inhibition is summarized in Fig. 1B. A non-linear least-squares fit of the Hill equation to the concentration-dependence data yielded an apparent K_d value of 1.91 ± 0.18 μM and a Hill coefficient of 1.06 ± 0.09.

H-89 inhibits Kir channels in rabbit small-diameter CASMCs

Previous reports have suggested that Kir channels are present in small-diameter cerebral and submucosal arterioles as well as in small-diameter coronary arterial smooth muscle cells in the pig, rabbit, and rat [4,9–14]. To record the Kir channel, smooth muscle cells were isolated from small diameter coronary arteries (<100 μm, SCASMC) of rabbit. Kir currents were recorded in response to a voltage step from −60 to −140 mV for 50 ms, followed by a depolarizing voltage ramp from −140 to +20 mV at a rate of 0.5 mV ms^{-1}. To increase the magnitude of the Kir currents, both extracellular and intracellular K^+ concentrations were maintained at 140 mM. The contribution of K_{ATP} and BK_{Ca} channels to the recorded currents was minimized by the inclusion of ATP (4 mM) in the pipette solution and iberiotoxin, a BK_{Ca} specific antagonist, in the bath solution.

As shown in Fig. 2A, H-89 markedly reduced the Ba^{2+}-sensitive Kir current in a dose-dependent manner (for example, 47.99 ± 6.70% inhibition at 3 μM, 74.00 ± 3.19 at 10 μM). The dose–response curves of H-89 activities on the Kir channels of SCASMCs are displayed in Fig. 2B. A non-linear least-squares fit of the data yielded a K_d value of 3.78 ± 0.37 μM and a Hill coefficient of 1.22 ± 0.05.

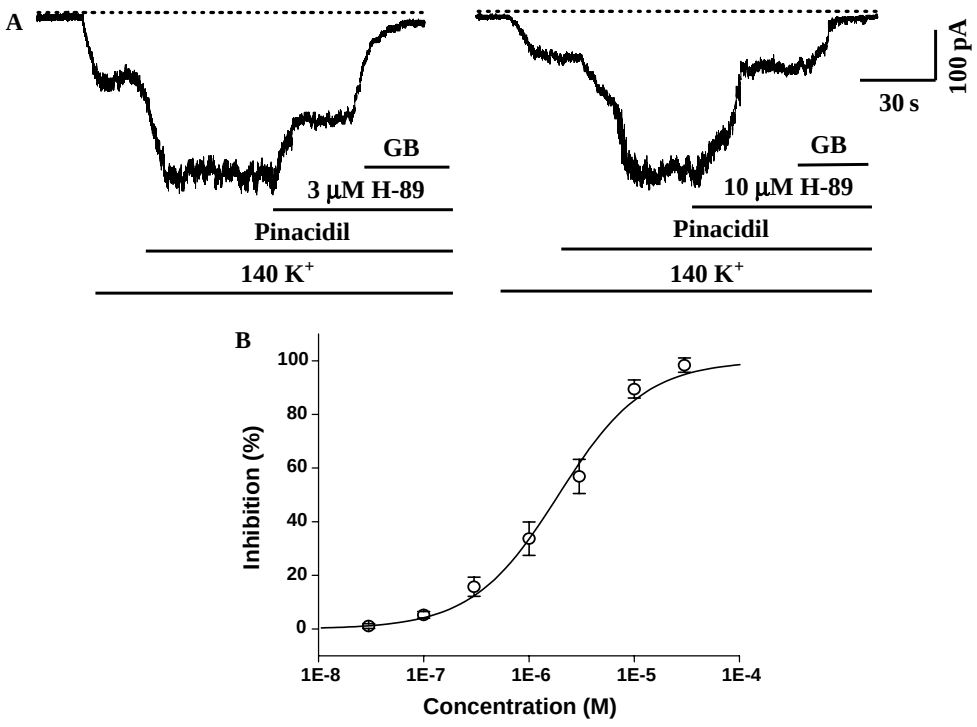

Fig. 1. H-89 inhibits pinacidil-induced K_{ATP} currents. Recording of whole-cell currents from a cell held at −60 mV, showing the pinacidil activation of glibenclamide (GB) sensitive K^+ currents in CASMCs. (A) The inhibition of the K_{ATP} currents in CASMCs by 3 μM and 10 μM H-89. (B) Concentration dependence of the inhibition of the K_{ATP} currents by H-89 (all $n = 4$). In all recordings, the dashed line indicates the zero current level.

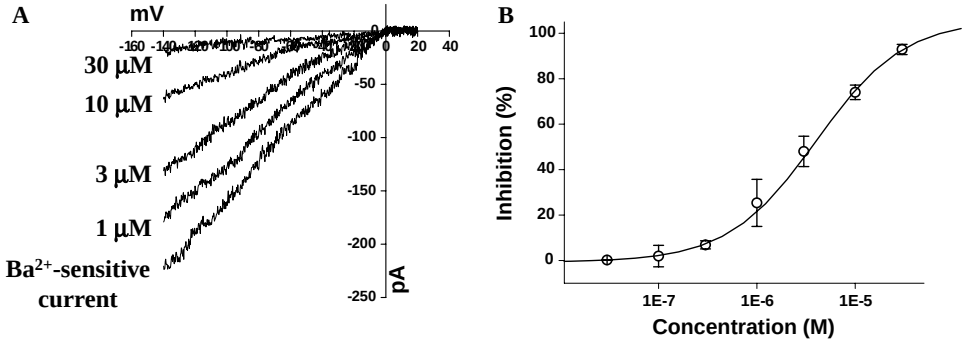

Fig. 2. Effect of H-89 on the whole cell Kir currents. The fraction of current that was Ba^{2+}-sensitive was determined by subtracting the current in the presence of 50 μM Ba^{2+} from that under the control condition. (A) Average current recorded in response to a voltage ramp (from −140 to +20 mV for 320 ms) superfused with control solution or solution containing H-89 (1, 3, 10, and 30 μM). (B) Average percent inhibition of the Kir current at −140 mV induced by different concentrations of H-89 in SCASMCs (all $n = 4$).

H-85 inhibits K_{ATP} and Kir channels

To investigate whether the inhibitory effect of H-89 on K_{ATP} and Kir currents is direct or through the inhibition of PKA, we employed H-85, an inactive form of H-89 on PKA inhibition (no inhibitory action on PKA). As shown in Fig. 3, 3 μM H-85 inhibits both K_{ATP} current and Kir currents, similar to the result of 3 μM H-89. Three micromolar H-85 inhibits K_{ATP} current by $52.67 \pm 8.02\%$ (Fig. 3A), and Kir current by $47.04 \pm 2.98\%$ (Fig. 3C), when 3 μM H-89 inhibits K_{ATP} current by $55.27 \pm 6.22\%$, and Kir current by $48.00 \pm 6.70\%$, respectively. These results suggest that H-89 did not inhibit K_{ATP} and Kir currents via an action on PKA but that this drug acted directly on K_{ATP} and Kir channels.

The effect of other PKA inhibitors on inhibition of K_{ATP} and Kir channel

To investigate whether the inhibition of K_{ATP} and Kir currents by H-89 resulted from an inhibition of PKA, we tested the effects of two other PKA inhibitors, Rp-8-CPT-cAMPs (10 μM), and KT 5720 (1 μM), on the K_{ATP} and Kir currents in CASMCs. Figure. 4 shows the effects of Rp-8-CPT-cAMPs and KT 5720 on the K_{ATP} currents. In contrast to the effect of H-89, Rp-8-CPT-cAMPs and

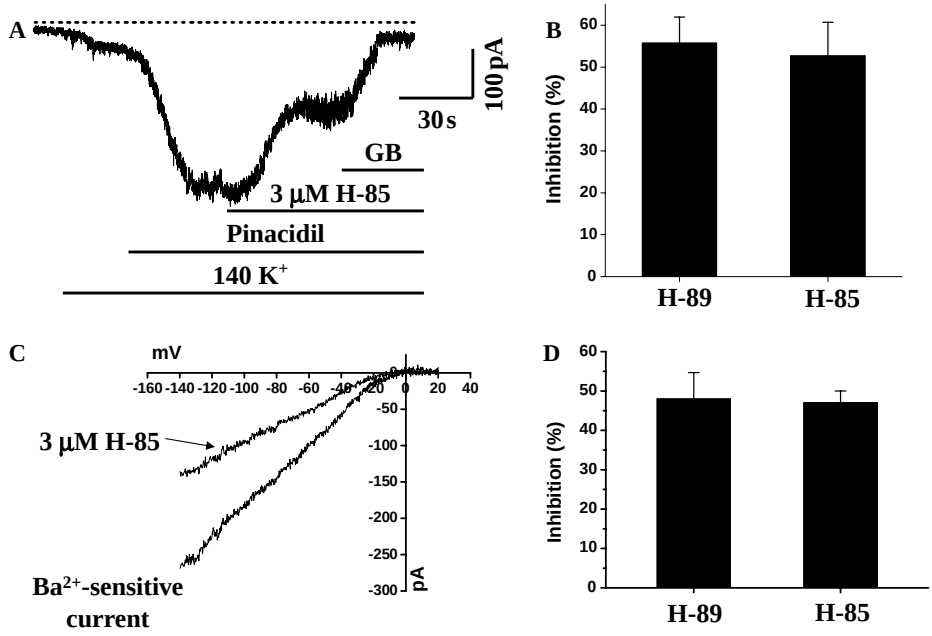

Fig. 3. Effect of H-85 on K_{ATP} and Kir currents. (A) Traces showing whole-cell currents activated by pinacidil, following the application of 3 μM H-85, and glibenclamide (GB) as indicated. (B) Summary of the inhibitory effects of H-85 and H-89 on K_{ATP} currents ($n = 4$). (C) The representative traces of Ba^{2+}-sensitive Kir currents in the absence and presence of 3 μM H-85. (D) Summary of the inhibitory effects of H-85 and H-89 on Kir currents ($n = 4$).

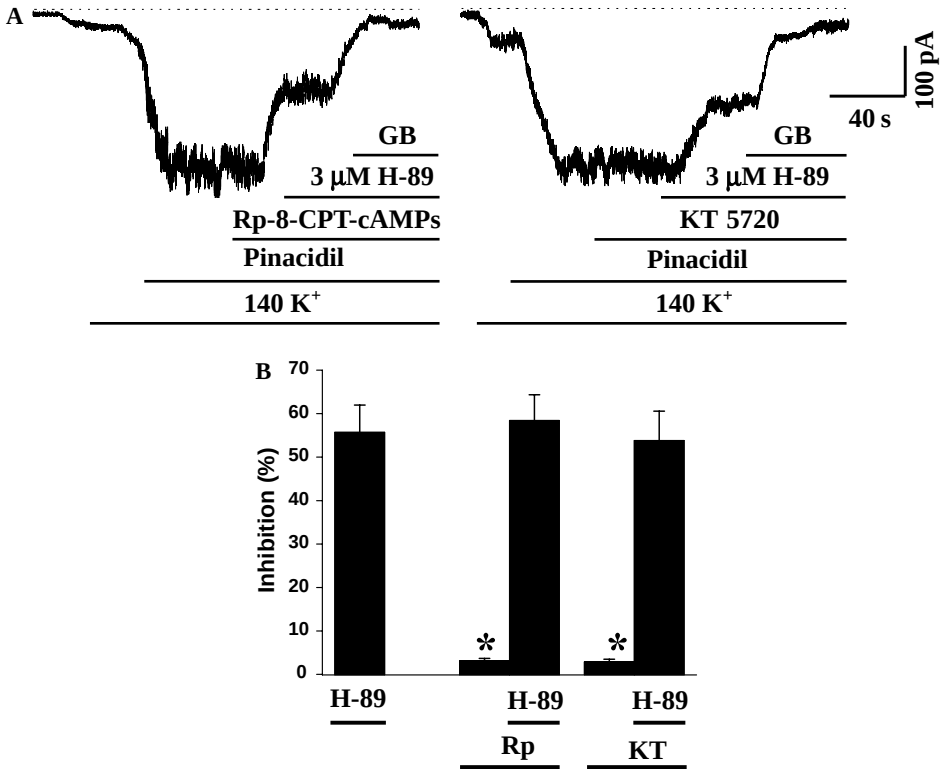

Fig. 4. Effects of Rp-8-CPT-cAMPs and KT 5720 on the H-89-induced inhibition of the K_{ATP} currents. (A) Traces showing whole-cell currents recorded from a cell exposed to pinacidil, Rp-8-CPT-cAMP (Rp, left panel) or KT 5720 (KT, right panel), H-89, and glibenclamide (GB) as indicated. (B) Summary of the effects of Rp and KT on the K_{ATP} currents in the absence and presence of H-89 (all $n = 5$).

KT 5720 failed to inhibit the K_{ATP} currents (Rp-8-CPT-cAMPs alone: $3.21 \pm 0.49\%$, KT 5720 alone: $2.88 \pm 0.61\%$ inhibition, respectively) and did not significantly alter the effect of 3 μM H-89 on the K_{ATP} currents (Rp-8-CPT-cAMPs + H-89: $58.44 \pm 5.90\%$, KT 5720 + H-89: $53.84 \pm 6.74\%$ inhibition, respectively). Similarly, the

application of Rp-8-CPT-cAMPs and KT 5720 did not affect the Kir current when applied alone (Rp-8-CPT-cAMPs alone: $5.02 \pm 0.64\%$, KT 5720 alone: $2.89 \pm 0.27\%$ inhibition, respectively) and did not significantly alter the effect of 3 μM H-89 on the Kir current (Rp-8-CPT-cAMPs + H-89: $45.72 \pm 7.21\%$, KT 5720 + H-89: $49.35 \pm 5.89\%$ inhibition, respectively) as shown in Fig. 5. These results suggest that H-89 does not inhibit K_{ATP} and Kir currents by acting on PKA, but rather, by acting directly on the K_{ATP} and Kir channels.

Discussion

The reduction of K_{ATP} and Kir currents seems not to be through influence of PKA but to be directly on K_{ATP} and Kir channels due to the following reasons: (1) previous report suggested that the vasodilator-induced increase in K_{ATP} currents was reduced by PKA inhibitors. However, K_{ATP} currents activated by the K_{ATP} channel opener pinacidil, thought to act directly on the K_{ATP} channel, were not affected by PKA inhibitors [3]. In our results, however, unlike other PKA inhibitors, H-89 reduced pinacidil-induced K_{ATP} and Ba^{2+}-sensitive Kir currents. Furthermore, H-85, which has a similar structure of H-89 and is considered as an inactive form of H-89, showed the similar effects on K_{ATP} and Kir currents. These results suggested that H-89 may cause direct inhibition of K_{ATP} and Kir channels. (2) Two other PKA inhibitors, Rp-8-CPT-cAMPs and KT 5720, which are structurally different from H-89, had no effect on K_{ATP} and Kir channels by themselves, nor did they modify the inhibition of K_{ATP} and Kir currents by H-89. These results strongly suggest that H-89 directly interacts with K_{ATP} and Kir channels, and inhibits these currents independently of PKA. (3) The effect of H-89 occurred rapidly (within 1 min) compared with the expected time course via PKA. The short exposure time required to reach steady-state inhibition is not simply explained by the inhibition of PKA activity.

Several subtypes of K^+ channels, including voltage-dependent K^+ (K_V), BK_{Ca}, K_{ATP}, and Kir channels, have been identified in cardiovascular (e.g., coronary) smooth muscle cells [15]. Among these channels, K_{ATP} channels play an important role in the regulation of the resting membrane potential, thereby maintaining vascular tone in coronary arterial smooth muscle cells [16]. Since, the activation of K_{ATP} channels leads to membrane hyperpolarization, vasodilation, and a decrease in vascular resistance. It has been suggested that Kir channels, which have been detected only in small-diameter cerebral and coronary arterial smooth muscle cells, also contribute to the resting tone in coronary and middle cerebral artery, as increase of moderate external K^+ concentration leads to vasodilation, and Ba^{2+} caused constriction of the vascular artery at resting tone [4,17]. In the present study, we found that K_{ATP} and

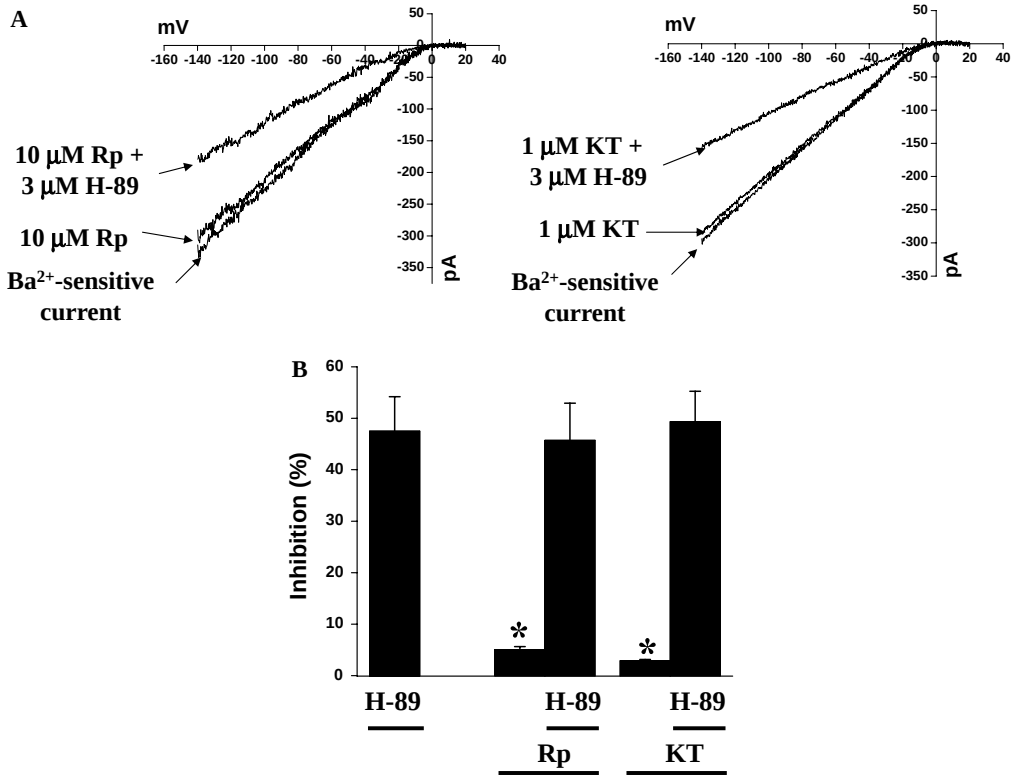

Fig. 5. Effects of Rp-8-CPT-cAMPs and KT 5720 on the H-89-induced inhibition of the Kir currents. (A) Traces show Ba^{2+}-sensitive currents recorded in a cell exposed to Rp-8-CPT-cAMP (Rp, left panel) or KT 5720 (KT, right panel), and H-89 as indicated. (B) Summary of the effects of Rp and KT on the Kir currents in the absence and presence of H-89 (all $n = 4$).

Kir channels were affected by H-89 independently of PKA. Therefore, we advocate caution when using H-89 in the functional studies in coronary arteries.

All members of the H series of chemicals, which act as protein kinase inhibitors, have 5-isoquinolinsulfonamide [18,19] as a key function group, and as a result of this common structure, are able to block ion channels that contain an environment similar to that of the protein kinase active site. The amino acids exposed in the pore cavity of ion channels are similar to those in the active site of protein kinases, i.e., Leu, Lys, Val, and Ala, and could form hydrogen bonds and/or van der Waals interactions with the H-series chemicals [5,20]. Indeed, in PKA active sites, Val-123 forms H-bond with H-series compounds, and Leu-49, Val-57, Ala-70, Lys-72, and Leu-173 form van der Waals interactions with H-89 [19]. However, the structures of K_{ATP} and Kir channels have not yet been identified with crystallography. Thus, we could explain the inhibitory effects of H-89 on K_{ATP} and Kir channels with qualitative analysis using the amino acid sequence of those proteins. Kir 6.1, which is consisting of K_{ATP} channels in the coronary arterial smooth muscle cells, has Leu-73 and Val-74 in M1 domain, and Ala-171, Val-172, and Leu-174 in M2 domain near the pore cavity [21]. Kir 2.1, which is believed to be Kir channels in the coronary smooth muscle cells [4], has Leu-85, Val-86, Leu-90, and Ala-91 in M1 domain, and Ala-178, Val-179, Ala-181, Lys-183, and Ala-184 in M2 domain near the cytosolic pore cavity [22]. Those amino acids are the candidates of interaction with H-series compound bringing out the inhibitory effects of H-89.

Despite their shared ability to inhibit channel activity, the H-series chemicals have variable efficiencies. For example, H-89 and H-85 block Na^+ channels with similar IC_{50} values, but higher concentrations of H-7 and H-8 are needed to block 50% of the Na^+ current [7]. Structural differences among the H-series chemicals, apart from isoquinolinesulfonamide group, probably account for the different efficiencies. Both H-89 and H-85 contain a butylenebenzyl group with a halogen ion, H-7 possesses a methylpoperazine group, and H-8 has a methyl group [23,24]. In the structure of H-89 and H-85, an alkyl chain with a double bond links the isoquinolinesulfonamide to the benzyl group, which contains a halogen ion in the *para* position, creating a polar–nonpolar structure similar to that of cell membrane components. Consequently, H-89 and H-85 easily interact with the membrane and place near the ion channels which are inserted into the membrane. Moreover, halogen ions of H-89 and H-85 may produce stronger van der Waals interactions with ion channel pores as described above.

Previous reports suggested that the K_i value of H-89 for PKA inhibition was 0.048 ± 0.0008 μM in in vitro system, the IC_{50} of H-89 for PKA inhibition was 135 nM by the protein kinase assay. Furthermore, the maximum effective concentration for PKA inhibition was 1 μM in Hepa cell [5,25,26]. Therefore, to block the effects of PKA, the concentration of H-89 ranged from 1 μM to 50 μM. In our report, we used 3 μM H-89. In the consideration of above reports, the application of 3 μM H-89 to inhibit the activity of K_{ATP} and Kir channels is physiologically reasonable. Therefore, caution is required when using H-89 in functional studies of modulation of ion channels by protein phosphorylation in coronary arterial smooth muscle cells.

Acknowledgments

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