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Short communication

Inhibition of Kv1.3 channels by H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide) independent of protein kinase A

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Abstract

The effects of H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide), a potent and selective inhibitor of protein kinase A (PKA), were examined on Kv1.3 channels stably expressed in Chinese hamster ovary (CHO) cells using the patch clamp technique. In whole-cell recordings, H-89 decreased Kv1.3 currents and accelerated the decay rate of current inactivation in a concentration-dependent manner with an IC_{50} value of 1.70 μM. These effects were completely reversible after washout. Intracellular infusion with PKA inhibitors, adenosine 3′, 5′-cyclic phosphorothioate-Rp (Rp-cAMPS) or protein kinase A inhibitor 5-24 (PKI 5-24) had no effect on Kv1.3 currents and did not prevent the inhibitory action of H-89 on the current. H-89 applied to the cytoplasmic surface also inhibited Kv1.3 currents in excised inside-out patches. These findings suggest that H-89 inhibits Kv1.3 currents independently of PKA.

Keywords: H-89; Kv1.3; Protein kinase A

1. Introduction

PKA is a family of protein kinases that specifically phosphorylate serine/threonine residues on the substrate protein. Voltage-activated ion channels are important target proteins for modulation by protein phosphorylation. Modulation of these channels by protein phosphorylation affects several physiological processes [1,2]. One of the methods to study the role of PKA in observed effects is to inhibit PKA activity by incubation with permeable and potent PKA inhibitors. For this purpose, membrane-permeable and selective PKA inhibitors are indispensable. Among these PKA inhibitors, H-89 has been reported to be a highly specific PKA inhibitor with little effect on the activity of PKC and other protein kinases [3]. However, little attention has been paid to the nonspecific effects of protein kinase inhibitors. This is significant given the importance of the finding that H-89 has nonspecific and direct inhibitory effects on sarcoplasmic reticulum Ca^{2+}-ATPase [4]. Up to the present, the direct inhibition of K^+ channels by PKC inhibitors was reported in several studies [5–7] but direct inhibition by PKA inhibitors has not been investigated.

We report for the first time that H-89 inhibits Kv1.3 channels, a member of the *Shaker*-type K^+ channel subfamily, by a mechanism which is independent of PKA.

2. Materials and methods

We used the stable CHO cell line expressing Kv1.3 as described previously [8]. Cells were cultured in Iscove's modified Dulbecco's medium (Life Technologies) supplemented with 10% fetal bovine serum, 0.1 mM hypoxanthine, 0.01 mM thymidine and 500 μg/ml G418 (Life Technologies). The Kv1.3 currents were recorded using the whole-cell and inside-out configurations of the patch-clamp

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Abbreviations: AMP-PNP, 5′-adenylyl-imidodiphosphate; CHO, Chinese hamster ovary; H-89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; PKA, protein kinase A; PKC, protein kinase C; PKI 5-24, protein kinase A inhibitor 5-24; Rp-cAMPS, adenosine 3′, 5′-cyclic phosphorothioate-Rp

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technique [9]. Currents were recorded using an Axopatch 200B amplifier (Axon Instruments) and analyzed by pClamp 6.03 software (Axon Instruments). For whole-cell recordings, the electrodes were filled with a solution containing (mM) 140 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 EGTA (pH 7.3 with KOH). This solution served as an external bath solution for inside-out patches. AMP-PNP (1 mM) was added to the bath solution for inside-out recordings and to the pipette solution for whole-cell recordings. The external bath solution for whole-cell recordings contained (mM) 140 NaCl, 5 KCl, 1.3 $CaCl_2$, 1 $MgCl_2$, 20 HEPES, and 10 glucose (pH 7.3 with NaOH). This solution was used as a pipette solution for inside-out patches. ATP was omitted from the solution in all experiments. During the recordings, the recording chamber (RC-13, Warner Instrument Corporation) was continuously perfused with test solution at a rate of 1 mL/min. H-89, AMP-PNP and PKI 5-24 were obtained from Calbiochem. Rp-cAMPS was purchased from Biomol. H-89 was dissolved in DMSO. The final concentration of DMSO never exceeded 0.1%; this concentration of DMSO had no effect on Kv1.3 currents. In all experiments, we used a pulse protocol consisting of a 200-msec depolarizing pulse from a holding potential of −80 mV to a test potential of +40 mV every 30 sec. Inhibition was assessed by measuring the current amplitude at the end of a 200-msec depolarizing pulse to +40 mV. The activation kinetics was calculated by fitting with a single exponential to the latter 50% of activation, which was considered to be the dominant time constant of activation (τ_{act}) [10]. Data were expressed as means ± SEM. Statistical significance was determined at the level of 0.05 using Student's *t*-test.

3. Results

Fig. 1A illustrates the effect of repeated treatments of H-89 (3 μM) on Kv1.3 currents stably expressed in CHO cells. When applied to the external bath solution in whole-cell recordings, H-89 reduced the current amplitude by 35.14 ± 4.32% (N = 5) within 3 min. Repeated application of H-89 to the same cell caused a rapid and reversible inhibition of the current amplitude. Although amplitudes of Kv1.3 showed a slow decline with time, the degree of inhibition did not decline. Fig. 1B shows traces of the currents obtained at a test potential of +40 mV in control and with 0.3, 3, and 10 μM H-89. H-89 decreased the Kv1.3 current in a concentration-dependent manner, and the peak current amplitude was affected much less than the current amplitude at the end of a 200-msec depolarizing pulse. H-89 did not affect activation kinetics (τ_{act}, control, 0.80 ± 0.14 msec; 1 μM H-89, 0.76 ± 0.16 msec, N = 6). Currents were measured at the end of a 200-msec depolarizing pulse to +40 mV to generate the concentration-response curve (Fig. 1C). A nonlinear least-squares fit of the data yielded an IC_{50} of 1.70 μM and a Hill coefficient of 1.17. Especially at higher concentrations, H-89 resulted in a marked acceleration of the apparent current inactivation. This effect was also concentration-dependent. Under control conditions, Kv1.3 current decay was well fitted to a monoexponential with a time constant of 160.57 ± 10.94 msec. After addition of 1, 3 and 10 μM H-89, Kv1.3 current decay was significantly accelerated and measured 149.12 ± 11.06, 125.10 ± 9.75 and 69.86 ± 4.35 msec, respectively (N = 5).

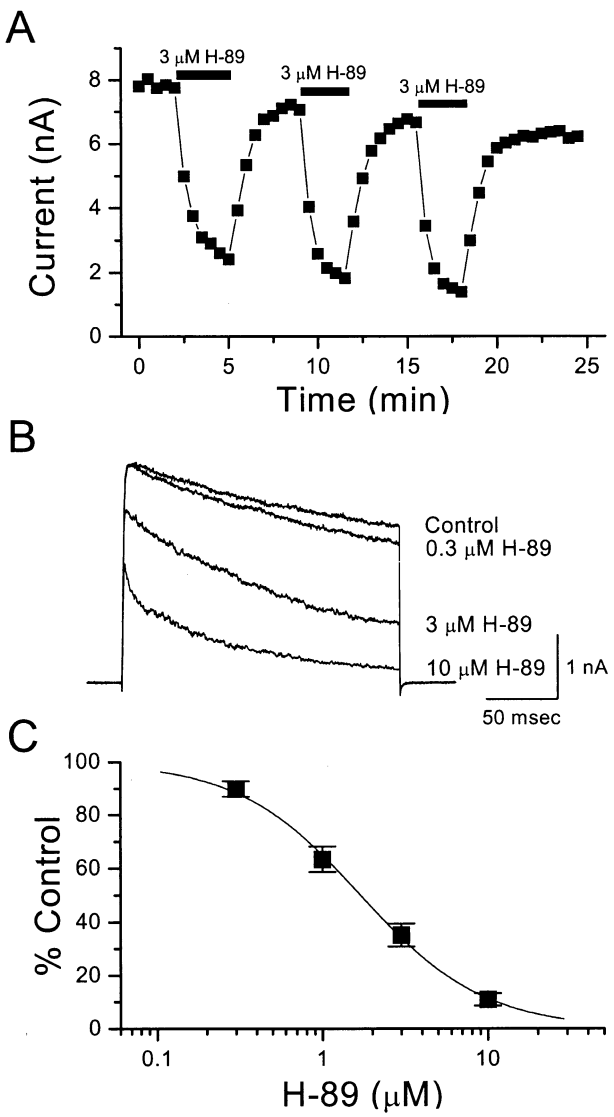

Fig. 1. Effects of H-89 on Kv1.3 currents in whole-cell recordings. Whole-cell currents were elicited by a 200-msec depolarizing pulse to +40 mV from a holding potential of −80 mV at 30-sec intervals. (A) The effect of repeated treatment of H-89 (3 μM) on Kv1.3 currents stably expressed in CHO cells. The bars indicate the treatment of 3 μM H-89. (B) Concentration-dependence of the effect of H-89 on Kv1.3. The traces of the currents obtained in the absence and presence of 0.3, 3 and 10 μM of H-89 are shown. (C) Concentration-response curve. Currents were measured at the end of a 200-msec depolarizing pulse to +40 mV to generate the concentration-response curve. The IC_{50} value for H-89 was 1.70 μM and the Hill coefficient was 1.17 (N = 5).

To investigate whether the inhibitory action of H-89 was due to its ability to inhibit PKA, we examined the effects of

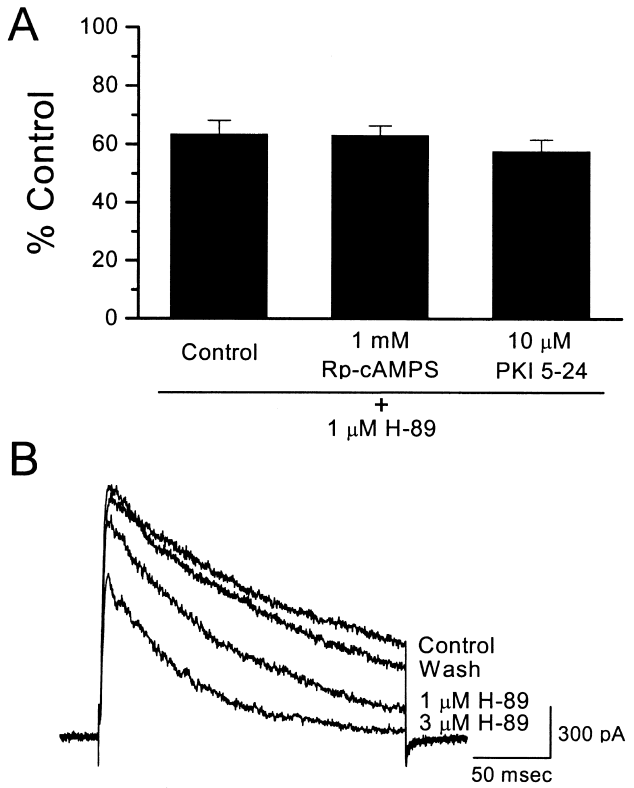

Fig. 2. (A) Effects of protein kinase inhibitors, Rp-cAMPS (N = 8) and PKI 5-24 (N = 7) on Kv1.3 currents in whole-cell recordings. Kv1.3 currents were evoked by a 200-msec depolarizing pulse to +40 mV from a holding potential of −80 mV. The drugs were included in the intracellular pipette solution. The protein kinase inhibitors alone did not affect the amplitudes of Kv1.3 currents during a 5-min recording period. (B) Effects of H-89 on Kv1.3 recorded from inside-out patches. Kv1.3 currents from inside-out patches were elicited by a 200-msec depolarizing pulse to +40 mV from a holding potential of −80 mV every 30 sec. The effects of H-89 (1, 3 μM) and washout of the drug are shown. The bath solution contained 1 mM AMP-PNP. Drug effects and washout took approximately 90 sec. Note the enhancement of current inactivation by H-89 as was the case for the whole cell currents.

other PKA inhibitors, Rp-cAMPS or PKI 5-24 (Fig. 2A). Because these compounds are not membrane-permeable, they were included in an intracellular pipette solution in whole-cell recordings. For fast intracellular infusion into the cytosol of cells, we used the patch pipette with a resistance of 1–2 MΩ when filled with a pipette solution. Over a 5-min period, cell dialysis with a pipette solution containing Rp-cAMPS (1 mM) and PKI 5-24 (10 μM) produced no effect on the amplitude and kinetics of Kv1.3. After pretreatment of Rp-cAMPS and PKI 5-24 for 5 min, H-89 (1 μM) reduced current amplitudes to 60.65 ± 1.85% (N = 8) and 63.00 ± 3.39% (N = 7) of control values, respectively. These values were not significantly different from those seen in the absence of drugs. We further examined the effects of H-89 on Kv1.3 currents using excised inside-out macropatches (Fig. 2B). The effects were similar to those observed in whole-cell recordings. H-89 (1 μM) decreased Kv1.3 currents to 39.37 ± 4.38% of control and accelerated the decay rate (83.08 ± 3.53 msec, control = 110.72 ± 6.96 msec, N = 6) of the Kv1.3 current in inside-out patches. Interestingly, the inhibition of currents in inside-out recordings was more potent than that in whole-cell recordings. The effects of H-89 were also rapid and largely reversible upon washout of the patch with drug-free solution.

4. Discussion

Kv1.3 is the major voltage-activated K^+ channel in T lymphocytes [11] and is also expressed in several regions of the brain [12]. The Kv1.3 gene sequences possess multiple consensus sites for PKA phosphorylation [13]. Furthermore, phosphorylation of Kv1.3 channels by PKA has been directly demonstrated in biochemical experiments [14]. Post-translational modification of Kv1.3 channels by PKA phosphorylation displays a variety of effects with short term T cell activation and long term T cell development [11]. However, the reported effects of PKA on Kv1.3 have varied widely among different studies ranging from inhibition, to no effect, to enhancement. For example, PKA activation has been reported to inhibit the activity of type *n* K^+ channels in the human Jurkat T cell line [15]. In human T lymphocytes, in contrast, native Kv1.3 channels were increased by PKA activation [16]. In the present study, we demonstrated that H-89, a selective and potent PKA inhibitor, produced a concentration-dependent and reversible inhibition of Kv1.3 channels. The following observations suggest that the effect of H-89 on Kv1.3 appears to be independent of PKA inhibition with a direct action on Kv1.3 channels. First, dialysis with PKA inhibitors, Rp-cAMPS and PKI 5-24 had no effect on Kv1.3 currents and did not prevent the inhibitory effect of H-89 on the current. Because the concentrations of PKA inhibitors, Rp-cAMPS and PKI 5-24, used in this study are sufficiently high to inhibit PKA activity completely, H-89-induced inhibition of Kv1.3 does not result from inhibition of PKA. Second, H-89 applied to the cytoplasmic side of the membrane showed similar effects in excised inside-out patch recordings. However, it is worth noting that PKA is strongly connected through its regulatory domain to some types of ion channels, even in excised inside-out patches [17,18]. In our experiments, the inhibitory effects of H-89 were observed under nonphosphorylating conditions (0 ATP and 1 mM AMP-PNP). Third, the effect of H-89 occurred rapidly compared with the time course of inhibition of PKA activity. For example, pretreatment of the cells with H-89 markedly inhibited forskolin-induced protein phosphorylation in a time-dependent manner [3]. These inhibitory effects were maximal at 24 hr. Therefore, the short exposure time to reach steady-state inhibition and the immediate reversibility upon washout are not simply explained by the inhibition of PKA activity. Taken together, these findings suggest that inhibition of the PKA pathway by H-89 appears not to mediate the inhibitory effect of H-89 on Kv1.3. Similarly, other nonspecific and

direct effects were demonstrated for H-89 on sarcoplasmic reticulum Ca^{2+}-ATPase in isolated ferret ventricular myocytes [4].

A limitation of our study is that we did not elucidate the mechanisms of action for H-89 on Kv1.3. In the present study, the most prominent effect of H-89 was to accelerate the rate of Kv1.3 current decay during the depolarizing pulse. This finding is consistent either with an open channel block or with acceleration of channel inactivation because the inactivation of Kv1.3 currents is normally minimal during a 200-msec depolarizing pulse to +40 mV. Furthermore, the time course of current activation was unaffected by H-89, which indicates that inhibition did not occur until the channel opened. Therefore, the characteristics of Kv1.3 inhibition by H-89 are similar to previous findings of open channel block of Kv1.3 by staurosporine in the same cells [6]. Interestingly, although H-89 and staurosporine have no obvious structural similarity, they exhibit similar IC_{50} values as inhibitors of Kv1.3 channels.

In summary, this report is the first to examine the effect of H-89 on the voltage-activated K^+ channel, Kv1.3. We find that H-89 potently inhibits Kv1.3 by a mechanism that is independent of PKA inhibition, and the concentrations required to inhibit Kv1.3 are within the range of those used in physiological experiments.

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