Direct block of cloned hKv1.5 channel by cytochalasins, actin-disrupting agents

Bok Hee Choi,1 Jung-Ah Park,1 Kyung-Ryoul Kim,1 Ggot-Im Lee,1 Yong-Tae Lee,1 Huhn Choe,2 Seong-Hoon Ko,2 Min-Ho Kim,3 Yeon-Ho Seo,3 and Yong-Geun Kwak1

Departments of 1Pharmacology, 2Anesthesiology, and 3Thoracic and Cardiovascular Surgery, Chonbuk National University Medical School, Chonju, Chonbuk, Republic of Korea

Submitted 13 September 2004; accepted in final form 25 March 2005

Choi, Bok Hee, Jung-Ah Park, Kyung-Ryoul Kim, Ggot-Im Lee, Yong-Tae Lee, Huhn Choe, Seong-Hoon Ko, Min-Ho Kim, Yeon-Ho Seo, and Yong-Geun Kwak. Direct block of cloned hKv1.5 channel by cytochalasins, actin-disrupting agents. Am J Physiol Cell Physiol 289: C425–C436, 2005. First published March 30, 2005; doi:10.1152/ajpcell.00450.2004.—The action of cytochalasins, actin-disrupting agents on human Kv1.5 channel (hKv1.5) stably expressed in Ltk− cells was investigated using the whole cell patch-clamp technique. Cytochalasin B inhibited hKv1.5 currents rapidly and reversibly at +60 mV in a concentration-dependent manner with an IC50 of 4.2 μM. Cytochalasin A, which has a structure very similar to cytochalasin B, inhibited hKv1.5 (IC50 of 1.4 μM at +60 mV). Pretreatment with other actin filament disruptors cytochalasin D and cytochalasin J, and an actin filament stabilizing agent phalloidin had no effect on the cytochalasin B-induced inhibition of hKv1.5 currents. Cytochalasin B accelerated the decay rate of inactivation for the hKv1.5 currents. Cytochalasin B-induced inhibition of the hKv1.5 channels was voltage dependent with a steep increase over the voltage range of the channel’s opening. However, the inhibition exhibited voltage independence over the voltage range in which channels are fully activated. Cytochalasin B produced no significant effect on the steady-state activation or inactivation curves. The rate constants for association and dissociation of cytochalasin B were 3.7 μM/s and 7.5 s−1, respectively. Cytochalasin B produced a use-dependent inhibition of hKv1.5 current that was consistent with the slow recovery from inactivation in the presence of the drug. Cytochalasin B (10 μM) also inhibited an ultrarapid delayed rectifier K+ current (IK,ur) in human atrial myocytes. These results indicate that cytochalasin B primarily blocks activated hKv1.5 channels and endogenous IK,ur in a cytoskeleton-independent manner as an open-channel blocker.

VOLTAGE-GATED K+ (Kv) channels represent a structurally and functionally diverse group of membrane proteins. Kv channels play an important role in determining the length of the cardiac action potential and therefore are the targets for antiarrhythmic drugs (6). Multiple Shaker-like K+ channels and β-subunit genes have been cloned from human myocardium, and they functionally contribute to electrical activity of the myocardium (7). One of these Shaker channels, Kv1.5, is known to be a cardiovascular-specific K+ channel isoforms identified to date, although it has been found in other tissues (7, 23, 24, 32). Human Kv1.5 (hKv1.5), cloned from human heart, forms the molecular basis for an ultrarapid delayed rectifier K+ current (IK,ur) identified in the human atrium (7, 8, 38). Therefore, hKv1.5 may be an important molecular target for the treatment of atrial tachyarrhythmias, which represent a major clinical problem with serious morbidity (5). Interestingly, cardiac Kv1.5 channels are coupled to an actin cytoskeleton that can regulate current density and channel localization (20). Specific disruptors of these cytoskeletons have been used in analyzing the functional roles of cytoskeleton, including the modulation of ion channel activities. However, the usefulness of cytoskeleton disruptors is limited by their nonspecific actions on other targets. Cytochalasins are a group of fungal metabolites that are related by structure and biological activity that inhibit a wide variety of cell movements through the disruption of actin filament (10, 31). Those agents have also been widely used to elucidate the functional roles of actin. However, the direct effect of cytochalasins on hKv1.5 has not been studied. In the present study, we have examined the effects of cytochalasins on the hKv1.5 channels that are stably expressed in Ltk− cells.

MATERIALS AND METHODS

Isolation of human atrial myocytes. Atrial appendage from patients who underwent aortocoronary bypass surgery was obtained with the written consent of the patients. The tissues were minced and placed in Ca2+-free Krebs-Henseleit solution for 5 min by continuous bubbling with 100% O2. The minced tissues were incubated in 10-ml enzyme solution [200 U/ml collagenase (CLSII, Worthington Biochemical, Freehold, NJ), 4 U/ml protease (type XXIV, Sigma)] for 45 min. The tissues were then transferred to 10 ml of fresh enzyme solution and observed every 15 min under an inverted microscope for optimum digestion. They were then transferred to a storage solution composed of 20 mM KCl, 10 mM KH2PO4, 10 mM glucose, 70 mM glutamic acid, 10 mM β-hydroxybutyric acid, 10 mM taurine, 10 mM EGTA, and 0.1% albumin, at pH 7.4 with KOH, and the cells were dissociated by pipetting.

Transfection and Ltk− cell culture. The method used to establish hKv1.5 expression in a clonal mouse Ltk− cell line is the same as that previously described (28, 30). The expression vector contains a dexamethasone-inducible murine mammary-tumor virus promoter that controls transcription of the inserted cDNA, and it also contains a gene-confering neomycin resistance that is driven by the SV40 early promoter. The cells used for the experiments in the present study displayed hKv1.5-specific mRNA expression after dexamethasone treatment, as evidenced by Northern blot analysis (32). The transfected cells were cultured in Dulbecco’s modified Eagle’s media (Life Technologies, Grand Island, NY) supplemented with 10% horse serum and 0.25 μg/ml G418 (a neomycin analog, Life Technologies) under a 5% CO2 atmosphere. The cultures were passed every 3 to 5 days with the use of trypsin. Before the experiments, the subconfluent cells were incubated with 2 μM dexamethasone for 12 h to induce the expression of hKv1.5 channels. The cells were removed from the dish with a rubber policeman. The cell suspension was stored at room temperature (20–22°C) and was used within 12 h for the experiments.

Fluorescence microscopy. Before being fluorescent stained, hKv1.5-transfected Ltk− cells were fixed for 10 min in 3.7% formaldehyde, 0.125 M Tris-HCl (pH 7.4), and 0.1% Tween 20, washed, and permeabilized with 0.1% Triton X-100 in PBS. The cells were incubated with secondary antibodies, washed, and mounted with Fluoromount-G.
aldehyde in PBS solution (pH 7.4), rinsed three times with PBS, permeabilized for 5 min in a 0.2% Triton X-100 in PBS solution at room temperature, and rinsed three more times with PBS. Phalloidin-rhodamin dye (Molecular Probes, Eugene, OR) was then applied at a concentration of 100 nM according to the manufacturer’s instructions. After 20 min of incubation at room temperature, excess dye was removed by being rinsed three times with PBS. Fluorescence images were acquired with a charge-coupled device camera (model DXM1200, Nikon; Tokyo, Japan) mounted on an optical microscope (model Axiolicht S100, Zeiss, Oberkochen, Germany). For a rhodamin-labeled phalloidin pretreatment experiment, phalloidin-rhodamin dye was directly applied to the culture media for 12 h, and the cells were fixed with 3.7% formaldehyde, followed by fluorescence measurement as described above.

Electrophysiological recordings. Experiments were performed in a small volume (0.5 ml) bath mounted on the stage of an inverted microscope (model TE300, Nikon) and the bath was perfused continuously at a flow rate of 1 ml/min. hKv1.5 currents in Ltk cells were recorded at room temperature (20–22°C) using the whole cell configuration of the patch-clip clamp technique (11) with a patch-clip amplifier (Axopatch-200B, Axon Instruments, Foster City, CA). Currents were sampled at 1 to 10 kHz after an anti-aliasing filtering was done at 0.5 to 5 kHz. Data acquisition and command potentials were controlled with pCLAMP 6.05 software (Axon Instruments). Junction potentials were zeroed with the electrode in the standard bath solution. Gigaohm seal formation was achieved by suction. After whole cell configuration was established, the capacitive transients were elicited by a symmetrical 10-mV voltage clamp in steps from –80 mV, and they were recorded at 50 kHz for the calculation of cell capacitance. Whole cell currents of 2–4 nA and series resistances of 2–3 MΩ were used for the analysis.

Solutions and drugs. The intracellular pipette filling solution contained (in mM) 100 KCl, 10 HEPES, 5 K$_2$BAPTA, 5 K$_2$ATP, and 1 MgCl$_2$, and was adjusted to pH 7.2 with KOH. The bath solution contained (in mM) 130 NaCl, 4 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, and 10 glucose, and was adjusted to pH 7.35 with NaOH. Phalloidin (10 μM, Sigma) was added to the bath solution or to the culture media for phalloidin-pretreatment experiments. Cytochalasin B and cytochalasin D (Sigma) were dissolved in dimethyl sulfoxide (DMSO, Sigma) and phalloidin was dissolved in ethanol to yield stock solutions of 10 mM, respectively. The concentrations of DMSO and ethanol in the final solutions were <0.1%, and these concentrations had no effect on hKv1.5 currents.

Pulse protocols and analysis. The holding potential was –80 mV, and the cycle time for the protocols was 20 s. The standard protocol to obtain current-voltage (I-V) relationship and activation curves consisted of 250-ms pulses that were imposed in 10-mV increments from –60 to +60 mV. The steady-state currents were obtained at the end of 250-ms depolarizations. Deactivating tail currents were recorded at –50 mV. The concentration-response curves were fitted with the following logistic equation using Origin 5.0 software (Microcal Software, Northampton, MA):

$$ y = 1/[1 + ([D]/IC_{50})^n] $$  

where $IC_{50}$ is the concentration of cytochalasin B resulting in 50% inhibition, [D] is the cytochalasin B concentration, and $n$ is the Hill coefficient. Interaction kinetics between the drug and channel was described on the basis of a first-order blocking scheme as described previously (29). The apparent rate constants of association ($k_+$), dissociation ($k_-$), and theoretical $k_d$ were obtained from the following equations:

$$ 1/\tau_d = k_+[D] + k_1 $$  

$$ k_d = k_-/k_+ $$

where $\tau_d$ is the drug-induced time constant, which was calculated from a single exponential fits to the inactivating current traces during depolarization to +60 mV. The activation curve was obtained from the ratio of tail current amplitudes measured immediately after the decay of the capacitive transients. The voltage dependence of the channel opening (activation curve) was fitted with a Boltzmann equation

$$ y = 1/[1 + \exp[-(V - V_{1/2})/k]] $$  

where $k$ represents the slope factor, $V$ is the test potential, and $V_{1/2}$ represents the voltage at which 50% of the channels are open. The steady-state inactivation curve was obtained using a two-pulse protocol; currents were induced with 250-ms depolarizing pulse of +60 mV with 5-s preconditioning pulses from –60 to +20 mV by increments of 10 mV. The experimental data were fitted to the following equation:

$$ (I - I_e)(I_{\text{max}} - I_e) = 1/[1 + \exp(V - V_{1/2}/k)] $$

in which $I_{\text{max}}$ represents the current measured at the most hyperpolarized preconditioning pulse, $I_e$ represents a nonzero current that is not inactivated at the most depolarized 20-s preconditioning pulse, and $V$, $V_{1/2}$, and $k$ represent the preconditioning potential, the half-inactivation point, and the slope factor, respectively. We eliminated the nonzero residual current by subtracting it from the actual value. The dominant time constant of activation was calculated by fitting a single exponential to the latter 50% of activation (3, 28). The time constant of deactivation was also determined by a single exponential fitting.

The results are expressed as means ± SE. Student’s t-test and ANOVA were used to calculate statistical significance. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Figure 1, A and B, shows superimposed current traces of the hKv1.5 channels expressed in mouse Ltk$^-$ cells under control conditions and in the presence of cytochalasin B (10 μM). The cells were held at –80 mV and the 250-ms depolarizing pulses from –60 to +60 mV in 10-mV steps were applied every 20 s. Under control conditions, depolarizations positive to –40 mV elicited the outward currents that progressively increased with further depolarizations (Fig. 1, A and C). The dominant time constant of activation was 1.3 ± 0.2 ms at +60 mV ($n = 17$). At +60 mV, after the current reached the maximum peak, it declined slowly during the maintained depolarization. Outward tail currents exhibited a time constant of deactivation of 25.1 ± 3.1 ms ($n = 17$), which was similar to that previously described (28, 30). Cytochalasin B (10 μM) did not affect the initial activation phase of the current: the dominant time constant of activation was 1.4 ± 0.2 ms at +60 mV ($n = 7$). However, the slow inactivation was markedly accelerated, resulting in an apparent decrease of the steady-state current amplitude obtained at the end of a 250-ms depolarizing pulse (Fig. 1, B and C). Cytochalasin B had a less inhibitory effect on the peak current amplitude than on the steady-state current amplitude: cytochalasin B (10 μM) inhibited the peak current and the steady-state current at +60 mV to 65.5 ± 3.2% (n = 7, $P < 0.05$) and 26.9 ± 5.2% of the control value (n = 7, $P < 0.05$), respectively. The inhibition of hKv1.5 appeared within 20 s after the application of the drug and reached a new steady state within 2 min. Washout of cytochalasin B recovered the current to 93.2 ± 3.5% ($n = 7$) of the control value within 3 min. Figure 1C shows average I-V relations of the steady-state hKv1.5 current under control conditions and in the presence of 10 μM cytochalasin B. Cytochalasin B inhibited the amplitudes of steady-state currents evoked at voltages over which
cytochalasin B were $-15.9 \pm 1.3$ and $-17.4 \pm 1.5$ mV ($n = 7$), respectively, and the slope factors were not significantly different ($k = 5.4 \pm 0.7$ mV for control and $k = 7.0 \pm 0.8$ mV for 10 $\mu$M cytochalasin B; $n = 7$).

We investigated another actin disruptor, cytochalasin A, which has the most similar structure to cytochalasin B among other cytochalasin drugs. As shown in Fig. 2, the lower concentration (3 $\mu$M) of cytochalasin A than that of cytochalasin B (10 $\mu$M) inhibited hKv1.5 currents in a very similar way as cytochalasin B did. Cytochalasin A did not affect the initial activation phase of the current: the dominant time constant of activation was $1.4 \pm 0.3$ ms at $+60$ mV ($n = 5$). However, the slow inactivation was markedly accelerated, resulting in an apparent decrease of the steady-state current amplitude obtained at the end of a 250-ms depolarizing pulse (Fig. 2, B and C): cytochalasin A inhibited the peak current and the steady-state current at $+60$ mV to $56.6 \pm 7.4\%$ ($n = 5$, $P < 0.05$) and $23.7 \pm 5.4\%$ of the control value ($n = 5$, $P < 0.05$), respectively. The inhibition of hKv1.5 appeared within 20 s after the application of the drug and reached a new steady state within 1 min. Washout of cytochalasin A recovered the current to $95.2 \pm 3.3\%$ ($n = 5$) of the control value within 2 min. Figure 1C shows average I-V relations of the steady-state hKv1.5 current under control conditions and in the presence of 3 $\mu$M cytochalasin A. Cytochalasin A inhibited the amplitudes of steady-state currents evoked at voltages over which hKv1.5 shows average I-V relations of the steady-state hKv1.5 current under control conditions and in the presence of 3 $\mu$M cytochalasin A. Cytochalasin A inhibited the amplitudes of steady-state currents evoked at voltages over which hKv1.5 was activated. Cytochalasin A did not affect the voltage dependence of the activation curve: the values of V$_{1/2}$ for the activation under control conditions and in the presence of cytochalasin A were $-16.1 \pm 2.4$ and $-18.0 \pm 2.7$ mV ($n = 5$), respectively, and the slope factors were not significantly different ($k = 5.7 \pm 0.8$ mV for control and $k = 6.9 \pm 0.7$ mV for cytochalasin A; $n = 5$).

Figure 3, A and B, show the superimposed hKv1.5 current traces produced by a 250-ms depolarizing pulse of $+60$ mV under control conditions and in the presence of various concentrations of cytochalasin B or A. Steady-state currents were measured at the end of 250-ms depolarizing pulses to obtain
the concentration-response curves (Fig. 3, C and D). Plots of steady-state currents at depolarizing pulses of 0, +30, and +60 mV as a function of cytochalasin B or A concentrations were fitted to the Hill equation. For the cytochalasin B, the IC50 values obtained at depolarizing pulses of 0, +30, and +60 mV were 4.1 ± 0.3, 4.5 ± 0.5, and 4.2 ± 0.4 μM, respectively (n = 5 for each experiment), which were not significantly different (ANOVA, P < 0.05), and the Hill coefficients obtained at depolarizing pulses of 0, +30, and +60 mV were not significantly different (1.1 ± 0.1, 1.0 ± 0.1, and 0.9 ± 0.1, respectively; n = 5 for each experiment). For the cytochalasin A, the IC50 values obtained at depolarizing pulses of 0, +30, and +60 mV were 1.4 ± 0.1, 1.4 ± 0.2, and 1.5 ± 0.1 μM, respectively (n = 5 for each experiment), which were not significantly different (ANOVA, P < 0.05), and the Hill coefficients obtained at depolarizing pulses of 0, +30, and +60 mV were not significantly different (1.4 ± 0.1, 1.4 ± 0.1, and 1.4 ± 0.1, respectively; n = 5, for each experiment). On the basis of the values of IC50 (Fig. 3), these results indicate that cytochalasin A is more potent than cytochalasin B in the inhibition of hKv1.5 currents.

Cytochalasin B is a well-known disruptor of actin filaments (10). To elucidate whether the actin cytoskeleton was involved in cytochalasin B-induced inhibition of hKv1.5 channels, we further examined the effects of other actin disruptors, such as cytochalasin D and cytochalasin J and an actin filament stabilizer phalloidin (22, 35) on the cytochalasin B-induced inhibition of hKv1.5 (Fig. 4). As shown in Fig. 4A, cytochalasin D (10 μM) started to inhibit hKv1.5 currents after 1 min of the application of the drug and showed the maximal effect within 3 min. Cytochalasin D itself reduced the steady-state current of hKv1.5 elicited by a pulse of +60 mV to 87.5 ± 2.2% (n = 5) of the control values with little effect on the inactivation rate: averaged current amplitudes were averaged, there was no significant difference between two groups: 3.4 ± 0.3 nA (n = 23) and 2.9 ± 0.3 nA (n = 18) without and with the preincubation of phalloidin for 12 h, respectively. However, the additional application of cytochalasin B (10 μM) to the cells preincubated with phalloidin for 12 h accelerated the inactivation of hKv1.5 current and decreased the steady-state current amplitude at +60 mV to 27.7 ± 3.7% of the control values obtained before the application of cytochalasin B (n = 5). Figure 4D shows the effects of 3 μM phalloidin in pipette solution on the cytochalasin B-induced inhibition of hKv1.5 currents. Twenty minutes after membrane rupture, which allows complete dialysis, the steady-state amplitude of hKv1.5 was not affected compared with the control measured immediately after membrane rupture: the

---

**Fig. 3.** Concentration-dependent inhibition of hKv1.5 current by cytochalasin B (A) and cytochalasin A (B). The current traces recorded with 250-ms depolarizing steps to +60 mV from a holding potential of −80 mV every 20 s under control conditions and in the presence of various concentrations of cytochalasins B and A. Only the depolarizing pulse was changed for the concentration-dependent test at 0 and +30 mV. The dotted lines represent zero current. Concentration-response relationship of the hKv1.5 inhibition by cytochalasin B (C) and cytochalasin A (D). Steady-state currents taken at the end of the depolarizing pulses of 0 (○), +30 (■), and +60 mV (▲) were normalized to the controls for constructing the concentration-response curve. Data were fitted with a Hill equation (Eq. 1). Data are expressed as means ± SE.
averaged current amplitudes of the hKv1.5 channel were 2.8 ± 0.3 and 2.6 ± 0.2 nA for the current amplitudes of hKv1.5 current measured immediately and 20 min after membrane rupture, respectively. With the addition of 10 μM cytochalasin B to the bath solution, the steady-state amplitude of hKv1.5 was decreased by 24.9 ± 4.7% of the control values measured immediately after membrane rupture (n = 5). Data are summarized in Fig. 4E. The effects of pretreatment with cytochalasin D, cytochalasin J, and phalloidin on the kinetics of activation and inactivation of hKv1.5 were summarized in Table 1. These results strongly indicate that the actin disruption is not involved in the cytochalasin B-induced inhibition of hKv1.5.

To obtain direct morphological evidence, we examined the effects of actin disruptors or stabilizer on the structure of actin cytoskeleton in the hKv1.5-expressing Ltk− cells by fluorescence staining of actin cytoskeleton. Figure 5A shows an actin cytoskeleton staining of actin cytoskeleton. Figure 5 was decreased by 24.9 

![Fig. 4](http://ajpcell.physiology.org/) The effects of cytochalasins D and J and phalloidin on the inhibition of hKv1.5 currents by cytochalasin B. Representative superimposed currents were produced by applying 250-ms depolarizing pulses from a holding potential of −80 to +60 mV every 20 s. A and B: the control current, the current recorded after a 3-min exposure to cytochalasin D (10 μM) or cytochalasin J (10 μM), and the current obtained 2 min after the additional application of cytochalasin B (10 μM) to cytochalasin D are shown. C: the control current recorded in the cells preincubated with phalloidin (10 μM) for 12 h, and the current obtained 2 min after the additional application of cytochalasin B (10 μM), are shown. For this experiment, a bath solution containing phalloidin (10 μM) was used. D: the control current recorded immediately after membrane rupture, current recorded 20 min after membrane rupture, and current measured by treatment of 10 μM cytochalasin B are shown. Phalloidin (3 μM) was included in pipette solution. E: the steady-state current amplitudes measured at the end of a 250-ms depolarizing pulse under each set of experimental conditions (A–D) were normalized to those of the each control. The data for the inhibition of hKv1.5 by cytochalasin B (10 μM) alone at +60 mV were selected from Fig. 1B. The dotted lines represent zero current. Data are expressed as means ± SE.

Table 1. Time constants of activation and inactivation for hKv1.5 channel in presence of various drugs

<table>
<thead>
<tr>
<th>Drugs</th>
<th>n</th>
<th>Activation</th>
<th>Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17</td>
<td>1.3 ± 0.2</td>
<td>180.6 ± 12.4</td>
</tr>
<tr>
<td>Cytochalasin D</td>
<td>5</td>
<td>1.3 ± 0.2</td>
<td>187.3 ± 14.1</td>
</tr>
<tr>
<td>Cytochalasin J</td>
<td>5</td>
<td>1.4 ± 0.3</td>
<td>179.1 ± 12.8</td>
</tr>
<tr>
<td>Phalloidin (12 h)</td>
<td>5</td>
<td>1.4 ± 0.2</td>
<td>189.8 ± 13.7</td>
</tr>
<tr>
<td>Phalloidin (pipette)</td>
<td>5</td>
<td>1.3 ± 0.3</td>
<td>183.3 ± 11.4</td>
</tr>
<tr>
<td>Cytochalasin A</td>
<td>5</td>
<td>1.4 ± 0.3</td>
<td>30.1 ± 3.7</td>
</tr>
<tr>
<td>Cytochalasin B</td>
<td>7</td>
<td>1.4 ± 0.2</td>
<td>35.7 ± 3.7</td>
</tr>
<tr>
<td>Cytochalasin B + cytochalasin D</td>
<td>5</td>
<td>1.3 ± 0.3</td>
<td>36.8 ± 3.2</td>
</tr>
<tr>
<td>Cytochalasin B + cytochalasin J</td>
<td>5</td>
<td>1.4 ± 0.2</td>
<td>34.6 ± 3.7</td>
</tr>
<tr>
<td>Cytochalasin B + phalloidin (12 h)</td>
<td>5</td>
<td>1.3 ± 0.2</td>
<td>34.8 ± 3.2</td>
</tr>
<tr>
<td>Cytochalasin B + phalloidin (pipette)</td>
<td>5</td>
<td>1.4 ± 0.3</td>
<td>33.8 ± 3.8</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of pulses. hKv1.5, human voltage-gated K⁺ channel 1.5; +, pretreatment of latter drugs. The time constants of activation and inactivation at +60 mV were obtained as described in the text. For the concentration for each drug, see Fig. 4.
actin filaments were fragmented. Furthermore, the effect of cytochalasin D was more potent than that of cytochalasin J in disruption of actin cytoskeleton or cell membrane. More than 50% of the hKv1.5-expressing Ltk<sup>−</sup> cells treated with cytochalasin D lost their morphology or were dead. To investigate whether the treatment of phalloidin for 12 h was sufficient for the drug to enter the cells and thus stabilize the actin cytoskeleton, rhodamin-labeled phalloidin was applied to the culture media for 12 h. Figure 5 shows the actin cytoskeleton in the treatment with 10 μM cytochalasin D for 20 min after treatment with 1 μM rhodamin-labeled phalloidin for 12 h. Most of the cells showed fluorescence, indicating that rhodamin-labeled phalloidin entered to the cells. In addition, the disruption of actin cytoskeleton in the cells treated with rhodamin-labeled phalloidin by cytochalasin D was greatly reduced compared with that in the cells treated with 10 μM cytochalasin D alone (Fig. 5D). Thus treatment of phalloidin for 12 h was sufficient for the drug to enter into the cells and stabilize the action cytoskeleton. In 12-h treatment with cytochalasins A, B, D, and J, >90% of the hKv1.5-expressing Ltk<sup>−</sup> cells lost their morphology or were dead, indicating a critical actin disruption caused by the long-term treatment with these drugs (data not shown). Taken morphological data together, these results indicate that cytochalasin B inhibits the hKv1.5 currents independent of actin disruption.

Figure 6 shows the voltage dependence of the cytochalasin B- or cytochalasin A-induced inhibition of hKv1.5 channel. In the presence of cytochalasin B (10 μM) or cytochalasin A (3 μM), the blockade increased steeply between −30 and 0 mV, which corresponds to the voltage ranges of the channel opening (28). These data suggest that cytochalasin B or cytochalasin A binds primarily to the open state of the hKv1.5 channel. However, there was no additional inhibition of hKv1.5 by cytochalasin B or cytochalasin A in the ranges of voltages between 0 and +60 mV where the channels are fully activated. The linear curve fitting to the data points positive to 0 mV (Fig. 6, A and B, solid lines) yielded a value approximately equal to zero for the slope of the line: 27.8 ± 3.2% of the control value at 0 mV and 28.1 ± 2.7% of the control value at +60 mV (n = 5, ANOVA, P < 0.05) for the treatment of 10 μM cytochalasin B, and 25.8 ± 3.5% of the control value at 0 mV and 24.5 ± 3.1% of the control value at +60 mV (n = 5, ANOVA, P < 0.05) for the treatment of 3 μM cytochalasin A. The voltage dependence of the inactivation time constant (τ) is plotted in Fig. 6C. The time constants of the decay phase of hKv1.5 in the presence of 10 μM cytochalasin B or 3 μM cytochalasin A were significantly reduced compared with the controls, but this effect did not vary over the voltage ranges between +20 and +60 mV (ANOVA, P < 0.05): 177–201 ms for the control, 35–40 ms for 10 μM cytochalasin B, and 27–31 ms for 3 μM...
cytochalasin A. These results indicate that cytochalasin B- or cytochalasin A-induced inhibition of hKv1.5 channels is voltage independent over the voltage ranges in which channels are fully activated.

The kinetics between cytochalasin B and hKv1.5 channel were further investigated (Fig. 7). The effect of cytochalasin B on the voltage dependence of steady-state inactivation was examined using a typical two-pulse protocol under control conditions and in the presence of cytochalasin B (Fig. 7A). Under the control conditions, $V_{1/2}$ and $k$ showed no changes measuring $-29.9 \pm 0.7$ and $6.0 \pm 0.4$ mV ($n = 4$), respectively. These results indicate that cytochalasin B does not interact with hKv1.5 channel in the inactivated state.

Cytochalasin B accelerated the hKv1.5 current decay in a concentration-dependent manner (Fig. 3A). The traces of current decay at each concentration (1, 3, and 10 $\mu$M) of cytochalasin B were well fitted to a single exponential function that yielded a time constant for hKv1.5 current inhibition ($\tau_D$). A plot of the reciprocal of $\tau_D$ at $+60$ mV vs. each concentration yielded $k_{-1}$ of $3.7 \pm 0.4$ M/s and $k_{+1}$ of $7.5 \pm 0.5$ s$^{-1}$ ($n = 7$) (Fig. 7B). On the basis of the first-order interaction between cytochalasin B and hKv1.5, the theoretical $K_D$ value derived by $k_{-1}/k_{+1}$ yielded $2 \mu$M. Although the derived $K_D$ of $2 \mu$M is independent of the apparent IC$_{50}$ of $4.2 \pm 0.4$ M (at $+60$ mV) obtained from the concentration-response curve shown in Fig. 3C, the two values were reasonably close, suggesting that cytochalasin B interacts with hKv1.5 channel by a simple one-to-one reaction.

The use-dependent inhibition of hKv1.5 by cytochalasin B is shown in Fig. 7C. Under control conditions, the peak amplitude of the hKv1.5 current decreased by $3.8 \pm 0.4$ ($n = 5$), $7.4 \pm 0.5$ ($n = 5$), and $10.7 \pm 1.1$% ($n = 5$) after 20 repetitive 125-ms depolarizing pulses of $+60$ mV at frequencies of 1, 2, and 3 Hz, respectively. In the presence of cytochalasin B (10 $\mu$M), the peak current amplitude was not affected significantly at the first pulse, indicating that there is no tonic inhibition by cytochalasin B. The subsequent peak amplitude of hKv1.5 progressively decreased by $7.2 \pm 1.4$ ($n = 5$), $20.6 \pm 1.7$ ($n = 5$), and $32.6 \pm 1.8$% ($n = 5$) after 20 repetitive 125-ms depolarizing pulses of $+60$ mV at frequencies of 1, 2, and 3 Hz, respectively. A typical example of the recovery kinetics of hKv1.5 under control conditions and in the presence of cytochalasin B (10 $\mu$M) is shown in Fig. 7D. The recovery process was measured by a double-pulse protocol. Recovery from inactivation both under control conditions and in the presence of cytochalasin B (10 $\mu$M) was well fitted by a single exponential with recovery time constants of $132.7 \pm 16.5$ ms ($n = 5$) and $202.1 \pm 20.6$ ms ($n = 5$), respectively. The increased time constants of recovery from inactivation suggests that dissociation rate of cytochalasin B is lower than the transition rate between the open and closed (or resting) state under control conditions, which may explain the use-dependent inhibition (Fig. 7C).

To investigate the effects of cytochalasin B on endogenous hKv1.5 channels, the effect of cytochalasin on $I_{K,ur}$ currents in human atrial myocytes was investigated (Fig. 8). These currents display several similarities to those of hKv1.5 (7, 8, 38). For a measurement of $I_{K,ur}$, a prepulse was typically applied to inactivate 4-aminopyridine-sensitive transient outward K$^+$ current (18, 26). In the present study, a 100-ms prepulse to $+60$ mV was introduced to inactivate the transient outward K$^+$ current, followed by a 200-ms depolarizing pulses ranging from $-50$ to $+60$ mV after a 10-ms interval to record $I_{K,ur}$. Depolarizing pulses applied to atrial myocytes elicited $I_{K,ur}$, and this current showed outward rectification (Fig. 8A). These currents were substantially inhibited by 10 $\mu$M cytochalasin B (Fig. 8B). The I-V relation for $I_{K,ur}$ from Fig. 8, A and B, was shown in Fig. 8C. Cytochalasin B (10 $\mu$M) inhibited the steady-state current at $+60$ mV to $29.9 \pm 4.5$% of the control.
Fig. 7. Effects of cytochalasin B on kinetics of hKv1.5 currents. A: steady-state inactivation curves under control conditions (○; n = 4) and in the presence of 10 µM cytochalasin B (■; n = 4) were obtained using a two-pulse protocol, followed by fitting to Eq. 5. B: concentration-dependent kinetics of the cytochalasin B-induced inhibition of hKv1.5. The drug-induced time constants (τ_i) were obtained from a single-exponential function to the decaying traces of the hKv1.5 current. The reciprocal of drug-induced time constants obtained at +60 mV (with same pulse protocol as in Fig. 3A) were plotted vs. the cytochalasin B concentrations. The solid line represents the least-squares fit of the data to Eq. 2. k_+1 and k_-1 were obtained from the slope and intercept values of the fitted line. C: kinetic dependence of the hKv1.5 current by cytochalasin B (10 µM). Twenty repetitive 125-ms depolarizing pulses of +60 mV from a holding potential of −80 mV were applied at three different frequencies, 1, 2, and 3 Hz under control conditions (○, △, ▽), and in the presence of 10 µM cytochalasin B (■, ▲, ▼), respectively. The peak amplitudes of current at every pulse were normalized by the peak amplitudes of current obtained at the first number of pulse and then plotted vs. the pulse numbers. D: effect of cytochalasin B on the kinetics of hKv1.5 recovery from steady-state inactivation. The degree of recovery was measured by following a double-pulse protocol; the first prepulse of a 250-ms depolarizing potential of +60 mV from a holding potential of −80 mV was followed by the second identical pulse after increasing time intervals between 30 and 5,000 ms at −80 mV. Every cycle of the double-pulse protocol was 30 s. The peak currents elicited by the second test pulse were normalized against the peak currents obtained by the first prepulse and plotted as a function of various interpulse intervals under control conditions (○) and in the presence of 10 µM cytochalasin B (■). The curves were obtained by fitting the plotted data to a single exponential function. All data are expressed as means ± SE.

value (n = 5). As shown in Fig. 8D, the voltage-dependent inhibition of I_{K,ur} by 10 µM cytochalasin B was investigated by relative current from data presented in Fig. 8C. The blockade of I_{K,ur} by 10 µM cytochalasin B increased between −20 and 0 mV. However, there was no additional inhibition of I_{K,ur} by cytochalasin B in the range of voltages between +10 and +60 mV: 31.6 ± 4.8% of the control value at +10 mV and 29.9 ± 3.6% of the control value at +60 mV (n = 5, ANOVA, P < 0.05). These results suggest that cytochalasin B induces voltage-dependent inhibition of I_{K,ur} as did for hKv1.5 current.
DISCUSSION

Cytochalasins have been widely used for studying the functional roles of the cytoskeleton in cell systems because this agent causes the depolymerization of actin (10, 31). In this study, we have found that cytochalasin B directly inhibits hKv1.5 currents in a cytoskeleton-independent manner.

Several studies have demonstrated that K\textsuperscript+ channels can be regulated by cytoskeletal interactions. Actin-microfilament disruptors enhance the activities of ATP-sensitive K\textsuperscript+ channels in guinea pig cardiomyocytes (33). Cardiac Kv1.5 channels also couple to the actin cytoskeleton, which regulates current density and channel localization (20). Recently, it has been shown that modulation of Kv1.5 currents by protein kinase A requires an intact cytoskeleton (21). Specific disruptors for cytoskeletons have been used in analyzing the functional roles of the cytoskeleton, including modulation of ion channel activities. However, the usefulness of these disruptors is limited by nonspecific actions on other targets. Cytochalasins are a group of fungal metabolites that inhibit a wide variety of cellular activities through the disruption of actin filament (10, 31), and those drugs thus has been widely applied to elucidate the functional roles of actin.

In this study, we have found that cytochalasin B directly acts on hKv1.5, independent of actin-disruption pathways. The action of the cytochalasin B on hKv1.5 is very acute. Inhibition of hKv1.5 by cytochalasin B initiates rapidly within 20 s of its application, reaches a maximum effect within 2 min, and is also reversible within 3 min after washout. Cytochalasin B, if this drug acts through the disruption of actin filaments, modulates channel activity with a relatively slow time course of inhibition. For example, the application of 20 μM cytochalasin B progressively inactivated apical K\textsuperscript+ currents, leading to the complete channel inactivation in >10 min (37). Interestingly, Maruoka et al. (20) reported that cytochalasin B or cytochalasin D increased hKv1.5 current. This result is somehow opposite to our results. However, there is a big difference in the exposure time to the drugs. Those effects were observed only when exposed to cytochalasin B or cytochalasin D for >2 h. In our study, the time course of the inhibition of hKv1.5 currents by cytochalasin B does not correspond to the slow time course of channel modulation by actin disruption, suggesting that the actin-disruption is not involved in the inhibition of hKv1.5 currents by cytochalasin B.

Cytochalasin D is known to be much more potent than cytochalasin B in disruption of actin filament (35). Consistent with the Urbanik and Ware study (35), our results show that the effect of cytochalasin D on actin disruption and change of cell morphology in hKv1.5-expressing LLC-PK1 cells is stronger than that of cytochalasin B. Furthermore, cytochalasin J shows the similar effects with cytochalasin D on disruption of the actin cytoskeleton or changes in cell morphology in hKv1.5-expressing LLC-PK1 cells. However, the inhibition of hKv1.5 by cytochalasin D or cytochalasin J is much lesser than that by cytochalasin B: 12% and 15% inhibition by 10 μM cytochalasin D and cytochalasin J, respectively, and 73% inhibition by 10 μM cytochalasin B at depolarizing pulse of +60 mV. Furthermore, pretreatment with cytochalasin D or cytochalasin J does not affect the cytochalasin B-induced inhibition of hKv1.5 currents, nor modifies the cytochalasin B-induced kinetics of the current, whereas cytochalasin B apparently induces an accelerated inactivation of hKv1.5 (Table 1). These results strongly suggest that cytochalasin B acts on hKv1.5 channel regardless of actin disruption.

There are several previous reports (20, 21, 33, 37) showing the regulation of ion channels by cytoskeleton. Maruoka et al. (20) have reported that disruption of the actin cytoskeleton with cytochalasin B or cytochalasin D significantly increases Kv1.5-dependent K\textsuperscript+ currents in Kv1.5-expressing human embryonic kidney cells. Terzic et al. (33) have shown that cytochalasin B enhances K\textsubscript{ATP} channel activity. In contrast with these results, Wang et al. (37) have reported inhibition of K\textsuperscript+ channel by cytochalasin B. However, regardless of the down-regulation of K\textsuperscript+ channels by cytochalasin B, our results show that the cytochalasin B-induced inhibition of hKv1.5 (Table 1). This effect of cytochalasin D on actin disruption and change of cell morphology in hKv1.5-expressing LLC-PK1 cells is stronger than that by cytochalasin D or cytochalasin J, respectively, and 73% inhibition by 10 μM cytochalasin B at depolarizing pulse of +60 mV.
(Fig. 9). On the basis of the structure, cytochalasin B and cytochalasin A have a very similar structure because cytochalasin A is an oxidized derivative of cytochalasin B, whereas both drugs are structurally different from cytochalasin D and cytochalasin J. Interestingly, although these drugs are known to interfere actin polymerization, cytochalasin B and cytochalasin A have also been known to inhibit glucose transport across cell membrane (19, 34). These observations may indicate the importance of their chemical structure for their action on the transmembrane-type transporters or channels. Thus this structure-based drug mechanism may explain our results that cytochalasin B and cytochalasin A inhibits hKv1.5 channels, whereas cytochalasin D and cytochalasin J do not.

The cytochalasin B-induced inhibition of hKv1.5 is characterized by an acceleration of current inactivation. This kind of result can be explained in terms of an open-channel blocking mechanism (2, 9, 30, 36). The effects of cytochalasin B are also use dependent: the degree of current inhibition increased with repetitive depolarizations without tonic block. This phenomenon is also cited as evidence of an open-channel blocking mechanism. These results indicate that cytochalasin B directly blocks the open-state hKv1.5 channel by acting as a pore blocker. A high degree of inhibition with strong voltage dependence is observed in the voltage ranges of the channel opening (Fig. 6A). These results suggest that cytochalasin B preferentially binds to the open state of the channel. The blocking effect of cytochalasin B resembles the effects described previously for other charged drugs which act as open-channel blockers (27). However, voltage independence is also observed over the voltage ranges at which the channels are fully activated. Cytochalasin B is mainly in the uncharged form at the intracellular pH of 7.2 (the pH of the pipette solution), the interaction between the uncharged cytochalasin B and hKv1.5 channel is independent of the transmembrane electrical field. Although the open channel is blocked by the charged form of the drugs, the blocking of open channel produced by the uncharged form of a drug has also been observed previously (3, 4, 16, 40). Cytochalasin B has no effect on the voltage dependence of steady-state inactivation curve. There is a possibility that cytochalasin B preferentially binds to the hKv1.5 in the inactivated state and potentiates or stabilizes the inactivation of hKv1.5. However, in the present study, cytochalasin B produces no significant shift in the inactivation curve and no significant difference in the IC50 values obtained at different voltages. Furthermore, inactivation rate shows the voltage independence in the presence of cytochalasin B (Fig. 6C). Taken together, these results suggest that cytochalasin B does not bind to the inactivated state of the hKv1.5 channel. The summary of above results can be interpreted by the following kinetic scheme:

\[
\begin{align*}
C & \xrightarrow{k_+} O \\
I & \xrightarrow{k_-} OD
\end{align*}
\]

where C represents the simplified closed or resting state of the channel in a Hodgkin-Huxley model (four independent conformational changes: C0 ↔ C1 ↔ C2 ↔ C3) (12, 39), and this simplification is based on the fact that a depolarizing pulse of +60 mV, hKv1.5 opens rapidly with a dominant time constant of 1.3 ms at +60 mV. OD is the drug-bound open state (usually termed “blocked state”), I is the inactivated state, and [D] is the concentration of cytochalasin B. \(k_+\) and \(k_-\) have values of 3.7 \(\mu\)M/s and 7.5 s\(^{-1}\), respectively. However, we cannot completely rule out the possibility that cytochalasin B accelerates the intrinsic inactivation of hKv1.5 channels.

The current generated by hKv1.5 channels is known to be similar in voltage dependence, kinetics, and pharmacological sensitivity to \(I_{Kur}\) recorded in human atrial myocytes (38), dog

Fig. 9. Structures of cytochalasins.
ventricle (14), and rat atria (1). In fact, the hKv1.5 channel protein has been identified in human atrial and ventricular myocardium (23). However, electrophysiological studies have shown the absence of hKv1.5-like current in human ventricular myocytes (15, 17). These reports suggest that $I_{\text{k,ur}}$ is the native counterpart to hKv1.5 channels in the human atria (7, 28, 32, 38). The blockage of cardiac K$^+$ channels has been considered to prolong the action potential duration (6, 13, 25). Indeed, the selective blocking of hKv1.5-like current in human atrial myocytes results in a significant prolongation of the action potential duration (37). Our results show that cytochalasin B blocks a cloned cardiac channel (hKv1.5) expressed in Ltk$^-$ cells and an $I_{\text{k,ur}}$ in human atrial myocyte. Thus, cytochalasin B, like other hKv1.5 blockers, would be expected to suppress atrial tachyarrhythmias, and this drug could be a mother compound for development of new antiarrhythmic drugs specific for atrial tachyarrhythmias. However, the effect of cytochalasin B on the other cardiac channels should be examined to validate its usefulness and cardiac mechanisms.

In conclusion, this report is the first to detail the effects of cytochalasin B on voltage-gated K$^+$ channels. In the present study, it is concluded that cytochalasin B directly blocks one of cloned cardiac channel hKv1.5 and endogenous $I_{\text{k,ur}}$ regardless of actin disruption, which is concentration, time, voltage, and state dependent. The concentrations of cytochalasin B required to block hKv1.5 are lower than the concentrations that have been used in experiments designed to assess the role of actin filaments. Thus we strongly recommend caution in the use of these kinds of drugs in all experiments designed to determine the role of actin cytoskeleton. Alternatively, this study provides a pharmacological tool for the development of a specific ion channel blocker.

ACKNOWLEDGMENTS

We thank Dr. Michael M. Tamkun (Colorado State University) for the hKv1.5 cDNA and for critical comments on the manuscript, and Dr. Mie-Jae Im (Chonbuk National University) for comments.

GRANTS

This work was supported by Korea Research Foundation Grant Y00302.

REFERENCES