Direct Block by Bisindolylmaleimide of Rat Kv1.5 Expressed in Chinese Hamster Ovary Cells

BOK HEE CHOI, JIN-SUNG CHOI, SEONG-WHAN JEONG, SANG JUNE HAHN, SHIN HEE YOON, YANG-HYEOK JO, and MYUNG-SUK KIM

Departments of Physiology (B.H.C., J.-S.C., S.J.H., S.H.Y., Y.-H.J., M.-S.K.) and Biochemistry (S.-W.J.), College of Medicine, The Catholic University of Korea, Seoul, Korea

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ABSTRACT

The interaction of bisindolylmaleimide (BIM), widely used as a specific protein kinase C (PKC) inhibitor, with rat brain Kv1.5 (rKv1.5) channels stably expressed in Chinese hamster ovary cells was investigated using the whole-cell patch-clamp technique. BIM (I) and its inactive analog, BIM (V), inhibited rKv1.5 currents at +50 mV in a reversible concentration-dependent manner with an apparent Kd value of 0.38 and 1.70 μM, respectively. BIM (I) accelerated the decay rate of inactivation of rKv1.5 currents but did not significantly modify the kinetics of current activation. Other specific PKC inhibitors, chelerythrine and PKC 19–36, had no effect on rKv1.5 and did not prevent the inhibitory effect of BIM (I). The inhibition of rKv1.5 by BIM (I) and BIM (V) was highly voltage-dependent between −30 and 0 mV (voltage range of channel opening), suggesting that both drugs interact preferentially with the open state of the channel. The additional inhibition by BIM (I) displayed a voltage dependence (δ = 0.19) in the full activation voltage range positive to 0 mV, but was not shown in BIM (V) (δ = 0). The rate constants of association and dissociation for BIM (I) were 9.63 μM−1 s−1 and 5.82 s−1, respectively. BIM (I) increased the time constant of deactivation of tail currents from 26.35 to 45.79 ms, resulting in tail crossover phenomena. BIM (I) had no effect on the voltage dependence of steady-state inactivation. BIM (I) produced use-dependent inhibition of rKv1.5, which was consistent with the slow recovery from inactivation in the presence of drug. These results suggest that BIM (I) directly inhibits rKv1.5 channels in a phosphorylation-independent, and state-, voltage-, time-, and use-dependent manner.

The voltage-activated K+ channel Kv1.5 belongs to the Shaker-type K+ channel family and plays an important role in normal cardiac physiology, especially during cardiac action potential repolarization. The rat Kv1.5 channel was cloned from rat brain (Swanson et al., 1990) but it is also expressed in human heart (Tamkun et al., 1991). The rat brain Kv1.5 (rKv1.5) has multiple consensus sites for phosphorylation by protein kinase C (PKC), protein kinase A (PKA), and tyrosine kinase (Swanson et al., 1990; Tseng-Crank et al., 1990). This channel can be highly modulated by phosphorylation-involved signaling systems (Uebele et al., 1994; Li et al., 1996). Several inhibitors of protein kinases have been widely used to investigate the role of protein kinases in the signal transduction pathways. However, the possibility of direct action on ion channels by protein kinase inhibitors, especially PKC inhibitors, has been reported. Calphostin C, a selective inhibitor of PKC, has been shown to directly block L-type Ca2+ channels (Hartzell and Rinderknecht, 1996). It has also been reported that staurosporine, widely used as a potent PKC inhibitor, could inhibit muscarinic K+ channels (Lo and Breitwieser, 1994). Recently, we reported that staurosporine directly blocked cloned Kv1.3 without mediation through PKC or PKA inhibition or any other diffusible cytosolic molecules (Choi et al., 1999a). Another PKC inhibitor, bisindolylmaleimide (BIM) is structurally similar to staurosporine and has been known to have more potent selectivity of PKC inhibition over PKA or other protein kinases (Toullec et al., 1991). There are five types of BIM (I, II, III, IV, and V), which differ from each other in the potency of PKC inhibition. BIM (I) has the most potent activity of PKC inhibition among the five BIM types. As an inactive analog of BIM (I), BIM (V) has no activity of PKC inhibition and is useful as a negative control compound for PKC inhibition. In this study, we used these two types, BIM (I) and BIM (V), to investigate whether BIM can affect the properties of rKv1.5 channels stably expressed in Chinese hamster ovary (CHO) cells. This expression system of clone-specific channels is a very useful tool to study the functional and pharmacological characteristics of the ion channel of interest without the contamination of other ionic currents.

ABBREVIATIONS: rKv1.5, rat brain Kv1.5; BIM, bisindolylmaleimide; CHO, Chinese hamster ovary; PKA, protein kinase A; PKC, protein kinase C; IMDM, Iscove’s modified Dulbecco’s medium; DMSO, dimethyl sulfoxide; I-V, current-voltage.

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Our results suggest that BIM interacts with rKv1.5 channels in a PKC-independent manner and directly inhibits rKv1.5 currents. Furthermore, the inhibition kinetics of rKv1.5 currents by BIM is similar to a typical open channel blocker such as antiarrhythmic agents (Malayev et al., 1995; Valenzuela et al., 1996; Franqueza et al., 1998); namely, BIM preferentially interacts with the open state of the rKv1.5 channel.

Materials and Methods

Stable Transfection and Cell Culture. CHO cells (American Type Culture Collection, Rockville, MD) were maintained in Iscove’s modified Dulbecco’s medium (IMDM, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum, 0.1 mM hypoxanthine, and 0.01 mM thymidine. A rKv1.5 cDNA clone (Swanson et al., 1990) was subcloned into expression vector pcR3.1 (Invitrogen Corporation, San Diego, CA) using polymerase chain reaction. Sequences of the primer pair used in the polymerase chain reaction were as follows: forward primer, 5’-GACCATGAGATCCTCCG-3’; reverse primer, 5’-TGGTGTTGTAAACAGATG-3’. The plasmid DNA containing the rKv1.5 cDNA was purified by a DNA purification kit (Promega, Madison, WI). To produce transfection, 3 × 10⁵ CHO cells in 2 ml of IMDM were incubated with the rKv1.5 cDNA construct (1 μg) mixed with 3 μl of FuGENE6 (Boehringer Mannheim, Indianapolis, IN). The cells were incubated for 48 h under a 95% humidified air, 5% CO₂ environment at 37°C and subcultured by 1:10 dilution in IMDM containing 0.5 mg/ml G418 (Life Technologies). After 2 weeks, antibiotic-resistant clones were randomly selected and cultured in IMDM containing 0.2 mg/ml G418. The cultures were exchanged every 2 to 3 days with fresh IMDM containing 0.2 mg/ml G418 and passed every 4 to 5 days by use of a brief trypsin/EDTA treatment. The trypsin/EDTA-treated cells were seeded onto glass coverslips (diameter: 12 mm; Fisher Scientific, Pittsburgh, PA) in a Petri dish 24 h before use. For the electrophysiological experiments, cells-attached coverslips were transferred to a continually perfused recording chamber (RC-13; Warner Instrument Corporation, Hamden, CT).

Electrophysiological Recordings. The rKv1.5 current was recorded at room temperature (22–24°C) using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) with an Axopatch 1D patch clamp amplifier (Axon Instruments, Foster City, CA). Micropipettes were pulled from PG10165-4 glass capillary tubing (World Precision Instruments, Sarasota, FL) and had resistances of 1.5 to 3 MΩ when filled with internal pipette solution. Liquid junction potentials between external and pipette solution were offset before the pipette touched the cell. The micropipettes were gently lowered onto the potentials between external and pipette solution were offset before the to 3M (World Precision Instruments, Sarasota, FL) and had resistances of 1.5 MΩ. The effective series resistances were usually about 0.7 MΩ. Voltage drops, based on the calculated residual series resistances, were less than 2.2 mV. Sampling frequency was 5 kHz, and currents were filtered at 2 kHz (four-pole Bessel filter) before being digitized and stored on hard disk of a Digidata 1200A acquisition board (Axon Instruments)-equipped IBM pentium computer for subsequent analysis. All experimental parameters, such as pulse generation and data acquisition, were controlled using pClamp 6.03 software (Axon Instruments).

Solutions and Drugs. The bath solution contained: 140 mM NaCl, 5 mM KCl, 1.3 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, and 10 mM glucose, and was adjusted to pH 7.3 with NaOH. The internal pipette solution contained: 140 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 10 mM EGTA, and was adjusted to pH 7.3 with KOH. In the experiment involving PKC 19–36 (Research Biochemicals International, Natick, MA), it was directly added to the internal pipette solution. PKC 19–36 was dissolved in distilled water. BIM (I) and BIM (V) (Calbiochem, San Diego, CA) were dissolved in dimethyl sulfoxide (DMSO) to make stock solutions of 1 mM. Chelerythrine (Research Biochemicals International) was also dissolved in DMSO. The concentration of DMSO in the final dilution was less than 0.1%; this concentration of DMSO had no effect on rKv1.5 currents.

Data Analysis. For analysis, Origin 5.0 software (MicroCal Software, Inc., Northampton, MA) was used. Activation curves were fitted with a Boltzmann equation:

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

where \( k \) represents the slope factor, \( V \) the test potential and \( V_{1/2} \) the voltage at which the conductance was half-maximal. 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 (White and Bezanilla, 1985; Snyders et al., 1993). The steady-state voltage dependence of inactivation was investigated by using a two-pulse voltage protocol; currents were measured by a 250-ms test potential to +50 mV whereas 20-s pre-conditioning pulses were varied from −60 to 0 mV stepped by 10 mV in the absence and presence of drugs. The experimental points were calculated as shown in eq. 2a.

Normalized \( I = (I - I_c)/(I_{\text{max}} - I_c) \)

in which \( I_{\text{max}} \) represents the current measured at the most hyperpolarized pre-conditioning pulse and \( I_c \) represents a nonzero current that was not inactivated at the most depolarized 20-s pre-conditioning pulse. We eliminated this nonzero residual current by subtracting it from the actual value. The resulting steady-state inactivation data were fitted with a Boltzmann equation:

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

where \( V \) is the pre-conditioning potential, and \( V_{1/2} \) and \( k \) represent the potential corresponding to the half-inactivation point (in millivolts) and slope value (in millivolts), respectively. The time courses of the currents during test pulses and tail currents on repolarization were fitted with a single exponential function.

Interaction kinetics between drug and channel was described on the basis of a first order blocking scheme as described previously (Snyders and Yeola, 1995). From this concept, the apparent affinity constant \( K_d \) and Hill coefficient \( n \) were obtained by fitting concentration dependence data to the following equation:

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

where \( f \) is the fractional inhibition (\( f = 1 - I_{\text{drug}}/I_{\text{control}} \)) at test potential and where \( [D] \) represents various drug concentrations. The apparent rate constants of association (\( k_{\text{on}} \)) and dissociation (\( k_{\text{off}} \)) were obtained from the following equation:

\[ 1/\tau_d = k_{\text{on}}[D] + k_{\text{off}} \]

where \( \tau_d \) is the drug-induced time constant.

To investigate the voltage dependence of the fractional inhibition by the drug, we calculated the fractional inhibition at each test potential above the initial potential (~30 mV) of activation. Using the resulting data, the voltage dependence of the fractional inhibition was fitted with a Woodhull equation (Woodhull, 1973):

\[ f = [D]/[D] + K_d(0) \times \exp(-zSFV/RT) \]

where \( K_d(0) \) is the apparent affinity at 0 mV (the reference voltage), \( z \) is the charge valence of the drug, \( \delta \) is the fractional electrical distance (i.e., the fraction of the transmembrane electric field sensed
by a single charge at the receptor site), \( F \) is Faraday's constant, \( R \) is the gas constant, and \( T \) is the absolute temperature. In this study, we used 25.4 mV as the value of \( RT/F \) at 22°C.

Data were expressed as mean ± S.E. Student’s t test and ANOVA were used for statistical analysis. Statistical significance was considered at \( P < .05 \).

**Results**

**Concentration-Dependent Inhibition of rKv1.5 by BIM (I) and BIM (V).** Figure 1 illustrates the effects of BIM (I) and BIM (V) on rKv1.5 expressed in CHO cells. In the absence of any drug, rKv1.5 currents were rapidly activated and then slowly inactivated while a depolarizing pulse was maintained. The activation of the current proceeded with a sigmoidal time course, and the slow inactivation current declined about 13.01 ± 0.11% (\( n = 11 \)) compared with the peak current. In the presence of both drugs, the current decay was much faster than that observed without drugs, and steady-state currents measured at the end of the 250-ms depolarizing pulse decreased in a concentration-dependent manner (Fig. 1, A and B). The peak current amplitude was not affected by BIM (I) at the treated concentrations. BIM (V) also did not alter the peak current at low concentrations (0.3, 1 \( \mu \)M) but did at high concentrations (3, 10 \( \mu \)M). However, at high concentrations of BIM (V), the peak amplitude of current was affected much less than the steady-state current amplitude at the end of the 250-ms depolarizing pulse. When switched to solutions containing different concentrations of drugs, steady state was reached within 3 min. The washout of BIM (I) and BIM (V) by perfusion of drug-free solution was obtained within 3 min, and currents were recovered to 84.02 ± 0.04% (\( n = 6 \)) and 87.11 ± 0.11% (\( n = 5 \)) of control, respectively. A nonlinear least-squares fit of the Hill equation to the concentration-response data yielded an apparent \( K_d \) value and a Hill coefficient of 0.38 ± 0.02 \( \mu \)M and 1.10 ± 0.06 (\( n = 6 \)) for BIM (I) and 1.70 ± 0.08 \( \mu \)M and 1.79 ± 0.13 (\( n = 5 \)) for BIM (V) at +50 mV, respectively.

**The Effects of Other PKC Inhibitors on the Inhibition of rKv1.5 by BIM (I).** The rKv1.5 channel could be phosphorylated by PKC (Swanson et al., 1990; Tseng-Crank et al., 1990). To elucidate whether PKC was involved in BIM (I)-induced inhibition of rKv1.5, we further investigated the effects of other PKC inhibitors, such as chelerythrine in bath solution and the PKC pseudosubstrate peptide inhibitor, PKC 19–36 in pipette solution. Figure 2, A and C, shows the effects of chelerythrine on the inhibition of rKv1.5 by BIM (I). A 5-min exposure to 10 \( \mu \)M chelerythrine did not induce the inhibition of rKv1.5. After a 5-min exposure to 10 \( \mu \)M chelerythrine, BIM (I) inhibited the steady-state current of rKv1.5 measured at the end of the depolarizing pulse of +50 mV by 45.53%, which was not significantly different from the inhibition induced by BIM (I) (44.56%) in the absence of chelerythrine (see Fig. 1). Figure 2, B and C, shows the effects of 20 \( \mu \)M PKC 19–36 in pipette solution. Five minutes after membrane rupture, to allow for complete dialysis, the steady-state amplitude of rKv1.5 was not affected compared...
with the control measured immediately after membrane rupture. By adding 0.3 μM BIM (I) to the bath solution, the steady-state amplitude of rKv1.5 was decreased by 48.55%.

The lack of effects of chelerythrine and PKC 19–36 on BIM (I)-induced inhibition of rKv1.5 strongly indicates that BIM (I) directly inhibits rKv1.5 in a PKC-independent manner.

Voltage-Dependent Inhibition of rKv1.5 by BIM (I) and BIM (V).

Figure 3 shows the effect of BIM (I) on current-voltage (I-V) relations. Under control conditions, the I-V relationship was sigmoidal for depolarizing pulses between −30 and +10 mV and almost linear for depolarizing pulses greater than +10 mV (Fig. 3, A and C). As shown in Fig. 3, B and C, in the presence of 0.3 μM BIM (I), the inhibition of steady-state currents was observed in the entire voltage range over which rKv1.5 was activated. By plotting the relative inhibition (I_{BIM(I)}/I_{control}) versus potential (Fig. 3D), a high degree of inhibition with a strong voltage dependence was observed between −30 and 0 mV, which corresponds to the voltage range of the opening of channels. An additional low degree of inhibition with a weak but meaningful voltage dependence was detected in the voltage range between 0 and +50 mV in spite of rKv1.5 being fully activated at this voltage range. At a 0-mV depolarizing potential, 0.3 μM BIM (I) inhibited rKv1.5 currents by 36.31 ± 0.79% (n = 6). This inhibition continuously increased to 45.09 ± 1.41% (n = 6) at +50 mV. Under the assumption that BIM (I) interacts intracellularly with rKv1.5, we investigated this effect by a nonlinear curve fitting of the data at potentials positive to 0 mV using a simple Woodhull equation (see Materials and Methods). The solid line in Fig. 3D represents a fit curve which yielded a δ value of 0.19 ± 0.001 (n = 6). Figure 4 shows the effect of BIM (V) on I-V relations in the absence (Fig. 4A) and presence (Fig. 4B) of BIM (V). In the presence of 2 μM BIM (V), the inhibition of steady-state currents was observed in the full range of voltages in which rKv1.5 current was activated (Fig. 4C). By plotting the relative inhibition (I_{BIM(V)}/I_{control}) versus potential (Fig. 4D), a steep voltage dependence of inhibition, similar to BIM (I), was detected in the range of the activation of channels between −30 and 0 mV, suggesting that the rKv1.5 must open before the interaction between BIM (V) and rKv1.5 can take place. Interestingly, there was no additional inhibition of rKv1.5 by BIM (V) in the range of voltages between 0 and +50 mV. The nonlinear curve fitting (Fig. 4D, solid line) of the data at potentials greater than 0 mV yielded a slope value approximately equal to zero: 64.37 ± 0.0044% inhibition at 0 mV, 63.83 ± 0.0053% inhibition at +50 mV (n = 4). This means that the interaction between BIM (V) and rKv1.5 is independent of the membrane electric field (δ = 0).

Time Dependence of BIM (I)-rKv1.5 Interactions.
The voltage dependence of rKv1.5 inhibition by BIM (I) was similar to that of human Kv1.5 inhibition by antiarrhythmic agents (Snyders et al., 1992; Valenzuela et al., 1996; Longobardo et al., 1998). We investigated, in detail, the kinetics of inhibition of rKv1.5 currents by BIM (I). Under control conditions, the dominant time constant of activation was 1.01 ± 0.04 ms (n = 9) at a 250-ms depolarizing test pulse from −80 mV to +50 mV. In the presence of BIM (I), it was 0.90 ± 0.05 ms (n = 9), which indicated that the activation kinetics was not significantly modified by BIM (I). As shown in Fig. 1A, in the presence of BIM (I), the Kv1.5 current decay was accelerated in a concentration-dependent manner. The traces of current decay at each concentration (0.3, 1, and 3 μM) of BIM (I) were well fitted to a single exponential function that yielded a time constant for rKv1.5 current inhibition (τ_{i}). To minimize contamination by the time constant of the intrinsic slow inactivation current in the absence of BIM (I) from the time constant of decay currents induced by BIM (I), we did not use time constant value obtained at low concentration (0.1 μM) of BIM (I). A plot of the reciprocal of τ_{i} at +50 mV versus each concentration yielded an apparent association rate constant (k_{+1}) of 9.63 ± 0.29 μM^{-1} s^{-1} and an apparent dissociation rate constant (k_{-1}) of 5.82 ± 0.53 s^{-1} (Fig. 5). On
the basis of the first order interaction between BIM (I) and rKv1.5 (see Materials and Methods), the theoretical $K_a$ value derived by $k_{-1}$/$k_{+1}$ yielded 0.60 $\mu$M. Although the derived $K_a$ of 0.60 $\mu$M is independent of the apparent $K_a$ of 0.38 $\mu$M obtained from the concentration-response curve shown in Fig. 1A, the two values were reasonably close.

Figure 6 shows the superposition of the tail currents recorded at a 250-ms repolarizing return potential of $-40$ mV after a 250-ms depolarizing pulse of $+50$ mV from a holding potential of $-80$ mV under control conditions and in the presence of 0.3 $\mu$M BIM (I). Under control conditions, the tail current declined quickly with a time constant of 26.35 ± 2.77 ms ($n = 6$) and nearly completely deactivated during the 250-ms repolarizing potential of $-40$ mV. In the presence of BIM (I), the initial peak amplitude of tail current was reduced. The subsequent decline of the current was slower (45.79 ± 4.78 ms, $n = 6$, $P < .05$) than in control conditions, which resulted in the tail crossover phenomenon.

**Effects of BIM (I) on Steady-State Inactivation of rKv1.5.** The steady-state inactivation of rKv1.5 was investigated using a typical two-pulse protocol in the absence and presence of 0.3 $\mu$M BIM (I). As shown in Fig. 7, the potential $V_{1/2}$ ($\pm$ S.E.) of half-inactivation point and slope value ($k$) of the steady-state inactivation curves were $-32.20 \pm 0.08$ and $4.02 \pm 0.09$ mV for the control and $-34.30 \pm 0.12$ and $4.13 \pm 0.09$ mV for BIM (I), respectively ($n = 5$). The slight leftward shift of $V_{1/2}$ and a small change of $k$ by BIM (I) were not statistically significant. This lack of effect of BIM (I) on the voltage dependence of steady-state inactivation suggests that BIM (I) is unlikely to interact with the inactivated state of rKv1.5 channels.

**Use Dependence of BIM (I) Action on rKv1.5.** Figure 8 shows the use-dependent inhibition of rKv1.5 by BIM (I). Fifteen repetitive 125-ms depolarizing pulses of $+50$ mV from a holding potential of $-80$ mV were applied at two different frequencies, 1 and 2 Hz. Under control conditions, the peak amplitude of the rKv1.5 current decreased by 4.37 ± 0.58% ($n = 5$) at a frequency of 1 Hz and by 8.59 ± 0.94% ($n = 6$) at a frequency of 2 Hz in a weak frequency-dependent manner. In the presence of 0.3 $\mu$M BIM (I), the peak amplitude of rKv1.5 progressively decreased by 17.65 ± 0.88% ($n = 6$) and 29.88 ± 1.56% ($n = 6$) at 1 and 2 Hz, respectively, in a strong frequency-dependent manner. However, at the first pulse, the peak current amplitude was not significantly affected, indicating that there is no tonic inhibition by BIM (I).

**Effects of BIM (I) on the Kinetics of rKv1.5 Recovery from Steady-State Inactivation.** Figure 9 shows a typical example of the recovery kinetics of rKv1.5 in the absence and presence of 0.3 $\mu$M BIM (I). The recovery process was mea-
BIM has been widely used for studying signal transduction pathways in cell systems because it is more selective for PKC than staurosporine; BIM (I) has a half-inhibition value of 10 nM for PKC and 2 μM for PKA, whereas staurosporine has a value of 0.7 nM for PKC and 7 nM for PKA (Toulelc et al., 1991). Therefore, as a specific PKC inhibitor, BIM (I) may be useful in the investigation of the role of PKC in cellular responses. In this study, we found that BIM (I) directly inhibited rKv1.5 currents in a phosphorylation-independent manner although the rKv1.5 channel has multiple sites for phosphorylation by PKC, PKA, and tyrosine kinase (Swanson et al., 1990; Tseng-Crank et al., 1990). In our experiment, BIM (I) inhibited rKv1.5, resulting in a half-inhibition value of 0.38 μM. This value is much smaller than the half-inhibition reference value of 2 μM for PKA (see above), which indicates that the inhibitory effect on rKv1.5 by BIM (I) is not due to the inhibition of PKA. In addition, because BIM (I) has been known to poorly inhibit tyrosine kinase with a half-inhibition value of >50 μM, the possibility of the action of BIM (I) on rKv1.5 through mediating tyrosine kinase could be ruled out. We also tested the effect of rKv1.5 inhibition by BIM (V), which resulted in a half-inhibition value of 1.70 μM, although it has no activity on PKC inhibition. Moreover, other PKC inhibitors, including chelerythrine and PKC 19−36, had no effect on rKv1.5 by themselves, nor did they modify the inhibition of rKv1.5 by BIM (I). These results strongly suggest that BIM (I) directly interacts with rKv1.5 and inhibits its current independent of PKC inhibition.

A shallow voltage dependence of rKv1.5 inhibition by BIM (I) was demonstrated in the voltage range where conductance is saturated, but a similar relationship was not established with BIM (V). If a positively charged drug moves into the transmembrane electric field from the inside, then inhibition should increase on depolarization due to electrostatic repulsion between a positively charged BIM (I) and membrane depolarizing potential. This will occur in the voltage range where channels are in the opening state and should also occur over the voltage range where channels are fully activated. If the noncharged form of a drug accesses its binding site on rKv1.5 from the intracellular surface, an additional shallow voltage dependence of inhibition should not be observed in the voltage range where rKv1.5 channel is fully activated. In this study, this phenomenon is explained by a simple Woodhull model (eq. 5). BIM (I) is a weak base with a pK_\text{a} of 8.52. Therefore, at the intracellular pH of 7.3 (pH of the pipette solution), BIM (I) is mainly positive charged. The δ value of 0.19 for shallow voltage dependence (Fig. 3) indicates that the positively charged BIM (I) senses 19% of the applied transmembrane electrical field as referenced from the intracellular side. This value is similar to the δ values of 0.16 to 0.19 obtained in previous experiments with antiarrhythmic agents (Snyders et al., 1992; Valenzuela et al., 1996; Franqueza et al., 1998). On the other hand, despite a structural similarity between two drugs, BIM (V) has a low pK_\text{a} of −2.18 and is predominately its uncharged form at intracellular pH. Therefore, no additional inhibition was detected (δ = 0) in the voltage range where channels are fully activated although a high degree of inhibition with a strong voltage dependence was still detected in the voltage range of channels in the opening state (Fig. 4). The δ value of 0 suggests that the interaction between BIM (V) and rKv1.5 is independent of the transmembrane electric field.

We reported previously that staurosporine blocked the open state of Kv1.3 channels (Choi et al., 1999a). Staurosporine, BIM (I), and BIM (V) have an obvious structural similarity, including a conserved five-aromatic-ring motif, which may be the key structural determinant of these drugs in the blockade of Shaker-type K⁺ channels. BIM (I) and BIM (V) induced the inhibition of rKv1.5 in a concentration-dependent manner with K_d values of 0.38 and 1.70 μM, respectively. These different K_d values of BIM (I) and BIM (V) give an important clue in determining the binding site of rKv1.5. Structurally, BIM (I) has an additional hydrophobic alkyl chain group (Toulelc et al., 1991) in comparison with BIM (V). The different affinities of BIM (I) and BIM (V) indicate that hydrophobic interactions are important in determining the stability of the drug-channel interaction. The dissociation rate constant may correlate with a hydrophobic interaction; the faster dissociation rate constant may reflect a weaker hydrophobic component of interaction (Yang et al., 1995). We calculated the association and dissociation rate constants for BIM (V) using the same procedure described in Fig. 5, which yielded k_+ and k_− values of 13.04 ± 1.19 μM^{-1} s^{-1} (n = 4) and 17.77 ± 7.24 s^{-1} (n = 4), respectively. Thus, the estimated K_d (k_−/k_+) value for time-dependent inhibition by BIM (V) was 1.36 μM, which was similar to the K_d of 1.70 μM obtained from the concentration-dependent inhibition shown.
in Fig. 1. The association rate constant \((k_+)\) is 1.3 times faster than that of BIM (I) (9.63 \(\mu\)M\(^{-1}\) s\(^{-1}\)), whereas the dissociation rate constant for BIM (V) is 3.1 times faster than BIM (I) (5.82 s\(^{-1}\)). The main difference in dissociation rate constants reflects that BIM (I) may interact more stably with rKv1.5 by virtue of a stronger hydrophobic interaction than BIM (V). This correlates well with the difference in \(K_d\) value: the faster dissociation rate constant and lower potency. This interpretation was presented previously in other papers: TEA derivatives with squid potassium channels (Armstrong, 1971; Swenson, 1981); TEA analogs with Shaker channels (Choi et al., 1993); and terfenadine and quinidine with hKv1.5 (Snyders and Yeola, 1995; Yang et al., 1995). In this study, BIM preferentially interacts with the open state of the rKv1.5 channel (usually termed "open channel block") with the following results. First, BIM (I) accelerated the rate of rKv1.5 current decay. Second, BIM (I) did not affect the initial activation time course and the peak amplitude of the current at the onset of a depolarizing pulse. These results suggest that BIM (I) does not bind to the closed or resting state of rKv1.5. Third, blockade produced by BIM (I) was voltage-dependent and increased steeply in the voltage range of channel activation. Fourth, BIM (I) slowed the deactivation of the tail current, thus inducing a tail crossover phenomenon. This tail crossover phenomenon suggests an interaction between BIM (I) and the open state of the rKv1.5 channel (Snyders et al., 1992; Valenzuela et al., 1996; Choi et al., 1999b). Fifth, the inability of BIM (I) to shift the steady-state inactivation curve suggests that BIM (I) is unlikely to interact with the inactivated state of rKv1.5. The summary of the above results can be interpreted by the following kinetic scheme:

\[
\begin{align*}
C & \underset{k_+}{\rightleftharpoons} O \underset{k_-}{\rightleftharpoons} [B] \underset{k_d}{\rightarrow} OB
\end{align*}
\]

\(\text{Scheme 1.}\)

where \(C\) represents the simplified closed or resting state of the channel in a Hodgkin-Huxley model (four independent conformational changes: \(C_0 \leftrightarrow C_1 \leftrightarrow C_2 \leftrightarrow C_3\) (Hodgkin and Huxley, 1952; Zagotta et al., 1994) and this simplification is from the fact that on a depolarizing pulse of +50 mV, rKv1.5 opens rapidly with a dominant time constant of 1.01 ms. \(OB\) is the drug-bound open state (usually termed "blocked state"), \(I\) is the inactivated state, and \([B]\) is the concentration of BIM (I). \(k_+\) and \(k_-\) are the association and dissociation rate constants with the values of 9.63 \(\mu\)M\(^{-1}\) s\(^{-1}\) and 5.82 s\(^{-1}\), respectively. However, we cannot completely rule out the possibility that the acceleration of current decay in the presence of BIM may be due to a drug-induced acceleration in the conversion of open channels to the inactivated state.

In conclusion, this study is the first to investigate the direct effects of BIM PKC inhibitor on cloned rKv1.5 channels: a direct inhibition in a phosphorylation-independent, and in a state-, voltage-, time-, and use-dependent manner. The concentrations of BIM (I) required to block rKv1.5 are similar to those that have been used in physiological experiments designed to assess the role of PKC. Thus, much caution is required when using PKC inhibitor, BIM, for phosphorylation-involved ion channel modulation.

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References


Send reprint requests to: Sang-June Hahn, M.D., Dept. of Physiology, College of Medicine, The Catholic University of Korea, 505 Banpo-dong, Socho-gu, Seoul 137-701, Korea. E-mail: sjhahn@cmc.cuk.ac.kr