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European Journal of Pharmacology 483 (2004) 117–126
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Direct block by bisindolylmaleimide of the voltage-dependent K^+ currents of rat mesenteric arterial smooth muscle

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Received 5 May 2003; received in revised form 27 August 2003; accepted 10 October 2003

Abstract

We investigated the effect of bisindolylmaleimide (I), a widely used protein kinase C (PKC) inhibitor, on the voltage-dependent K^+ (Kv) currents of rat mesenteric arterial smooth muscle cells using the whole-cell patch-clamp technique. Bisindolylmaleimide (I) reversibly and dose-dependently inhibited the Kv currents with an apparent K_d value of 0.23 ± 0.001 μM. The blockade was apparently through the acceleration of the decay rate of the Kv currents. The apparent rate constants of association and dissociation for bisindolylmaleimide (I) were $17.9 \pm 1.6\ \mu M^{-1}\ s^{-1}$ and $4.1 \pm 1.5\ s^{-1}$, respectively. The inhibition of Kv current by bisindolylmaleimide (I) was steeply voltage-dependent between -30 and 0 mV (voltage range of channel activation). Bisindolylmaleimide (I) had no effect on the steady-state activation and inactivation of the Kv currents. Applications of trains of pulses at 1 or 2 Hz lead to a progressive increase in the bisindolylmaleimide (I)-blockade, and the recovery from bisindolylmaleimide (I)-block at -80 mV exhibited a time constant of 577.2 ± 52.7 ms. Bisindolylmaleimide (V), an inactive analogue of bisindolylmaleimide (I), similarly inhibited the Kv currents with an apparent K_d value of 1.48 ± 0.004 μM, but other PKC inhibitor chelerythrine little affected the Kv currents. These results suggest that bisindolylmaleimide (I) directly inhibits the Kv currents of rat mesenteric arterial smooth muscle cells independently of PKC inhibition, in a state-, voltage-, time- and use-dependent manner.

Keywords: K^+ channel; Bisindolylmaleimide; PKC (protein kinase C); Artery mesenteric; Smooth muscle; Patch-clamp; (Rat)

1. Introduction

Protein kinase C (PKC) is an enzyme that phosphorylates the hydroxyl group of serine and/or threonine (Ser/Thr) residues in various protein substrates, and this process is considered to be a fundamental regulatory mechanism involved in cellular growth, differentiation and immediate regulation of effector functions (Hug and Sarre, 1993; Nishizuka, 1995; Steinberg et al., 1995; Sugden and Bogoyevitch, 1995). In vascular tissue, PKC was suggested to be involved in vascular proliferation, pulmonary hypertension and reactive oxygen species-induced vascular smooth muscle cell apoptosis (Li et al., 1999; Weissmann et al., 1999; Watanabe et al., 2001). PKC has also been identified as a regulator of intracellular Ca^{2+} and contractility in vascular smooth muscles (Hori et al., 1993; Eto et al., 2001; Wesselman et al., 2001).

Ion channels are important targets for PKC-mediated signal pathways. Store-operated channels and voltage-dependent Ca^{2+} channels are known to be increased by PKC (Keef et al., 2001; Albert and Large, 2002), whereas voltage-dependent K^+ (Kv) channels were inhibited by PKC (Shimoda et al., 2001). The Kv currents contribute to the regulation of membrane potential in vascular smooth muscle cells (Nelson and Quayle, 1995; Archer et al., 1998). The inhibition of Kv currents depolarizes the smooth muscle membrane, which leads to the opening of voltage-dependent Ca^{2+} channels, and thus smooth muscle contraction. Regulation of Kv currents by PKC was reported in vascular smooth muscles and it has been proposed as one of the mechanisms of agonist-induced vasoconstriction (Clement-Chomienne et al., 1996; Standen and Quayle, 1998).

For investigating the role of PKC-mediated signal transduction, pharmacological inhibitors have been widely used

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doi:10.1016/j.ejphar.2003.10.028

both in vivo and in vitro studies. Bisindolylmaleimide I (GF109203X) is known as potent and selective PKC inhibitor (Toullec et al., 1991). However, their usefulness can be limited by non-specific actions on other targets than PKC. The direct effects on ion channels were reported in recent reports identifying inhibition by bisindolylmaleimide of rat Kv 1.5 channels (rKv1.5) expressed in Chinese hamster ovary (CHO) cells and acetylcholine-activated K^+ current in mouse atrial myocytes (Choi et al., 2000; Cho et al., 2001). Considering the significance of PKC in vascular function, the information about the non-specific actions of PKC inhibitors on vascular smooth muscles is necessary for the right interpretation of the experimental results using these substances. In the present study, we investigated the effect of bisindolylmaleimide (I) on the Kv current of rat mesenteric arterial smooth muscle cells and demonstrated that bisindolylmaleimide directly inhibited Kv current in state-, voltage-, time- and use-dependent manners, independently of PKC inhibition.

2. Materials and methods

2.1. Animals and cell preparation

Male Sprague–Dawley rats (7 weeks, 190–200 g) were obtained from Daehanbiolink (Korea). Rats were maintained at a constant temperature of 21 ± 1 °C and $55 \pm 5\%$ relative humidity on a normal 12-h light–dark cycle. Rats were stunned and rapidly exsanguinated by cutting the carotid arteries. Third order superior mesenteric arteries were carefully removed and placed in normal Tyrode solution. The arteries were dissected free from fat and connective tissue and cut into small pieces. Then, the arteries were transferred to digestion solutions. The first digestion solution was 3 ml of Ca^{2+}-free normal Tyrode solution containing papain (1 mg/ml; Sigma, USA), bovine serum albumin (1 mg/ml) and dithiothreitol (1 mg/ml). The second digestion solution contained collagenase (2 mg/ml; Wako, Japan) with same amount of bovine serum albumin and dithiothreitol in 3 ml of Ca^{2+}-free normal Tyrode solution. The arteries were incubated for 15 min in the first digestion solution, and then transferred to the second digestion solution for further 15-min incubation. After that, tissues were washed out twice using enzyme-free and Ca^{2+}-free solution for 15 min. Then, cells were isolated by gentle agitation with fire-polished glass pipette in Kraft–Brühe (KB) solution. The isolated cells were stored also in KB solution at 4 °C and used for the experiment at the same day.

2.2. Electrophysiological recordings

Once isolated, mesenteric arterial smooth muscle cells were subjected to patch-clamp experiments. We used the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) for the recording of Kv current with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA). Data were digitized with pCLAMP software 6.0.3 (Axon Instruments) at a sampling rate of 1–10 kHz, low-pass filtered at 1 kHz and stored on a computer. Voltage pulse generation was also controlled by pCLAMP software 6.0.3 (Axon Instruments). The patch pipettes were pulled from borosilicate capillaries (Clark Electromedical Instruments, Pangbourne, UK) using a puller (PP-83, Narishige, Japan). We used patch pipettes with a resistance of 2–3 MΩ when filled with the below pipette solutions. To allow adequate cell dialysis of the pipette solutions, recording were started at least 4–6 min after the formation of whole-cell configuration. All experiments were carried out at room temperature (22–25 °C).

2.3. Solutions and drugs

Solutions: Normal Tyrode (NT) solution contained (in mM): NaCl, 143; KCl, 5.4; NaH_2PO_4, 0.33; $CaCl_2$, 1.8; $MgCl_2$, 0.5; 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES), 5; glucose, 11; adjusted to pH 7.4 by NaOH. NT was used for the bath solution. The composition of pipette solution for recording of Kv currents was as follow (in mM): KCl, 140; NaCl, 5; Mg-ATP, 5; HEPES, 10; 1,2-bis(aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) 10; adjusted pH to 7.3 by KOH. KB solution contained (in mM): KCl, 50; K-glutamate, 50; KH_2PO_4, 20; $MgCl_2$, 3; taurine, 20; glucose, HEPES, 10; EGTA, 0.5; adjusted pH to 7.4 by KOH.

Drugs: bisindolylmaleimide (I), bisindolylmaleimide (V) and chelerythrine were purchased from Calbiochem (San Diego, CA). Bisindolylmaleimide (I) and bisindolylmaleimide (V) were dissolved in dimethyl sulfoxide (DMSO) and chelerythrine in distilled water to make stock solution. The final concentration of DMSO in the final dilution was less than 0.05%. All other chemicals were purchased from Sigma.

2.4. Statistics

Results are shown as the mean ± standard error of the mean. Student's *t*-test was used for the test of significance. Statistical significance was considered at $P < 0.05$. For data analysis, Origin 6.0 software (Microsoftware, Northampton, MA) was used.

3. Results

3.1. Bisindolylmaleimide (I) inhibited the Kv currents of rat mesenteric arterial smooth muscle cells

In order to record currents through Kv channels without the large conductance Ca^{2+}-activated K^+ (BK_{ca}) currents, tetraethylammonium 1 mM was added to the bathing

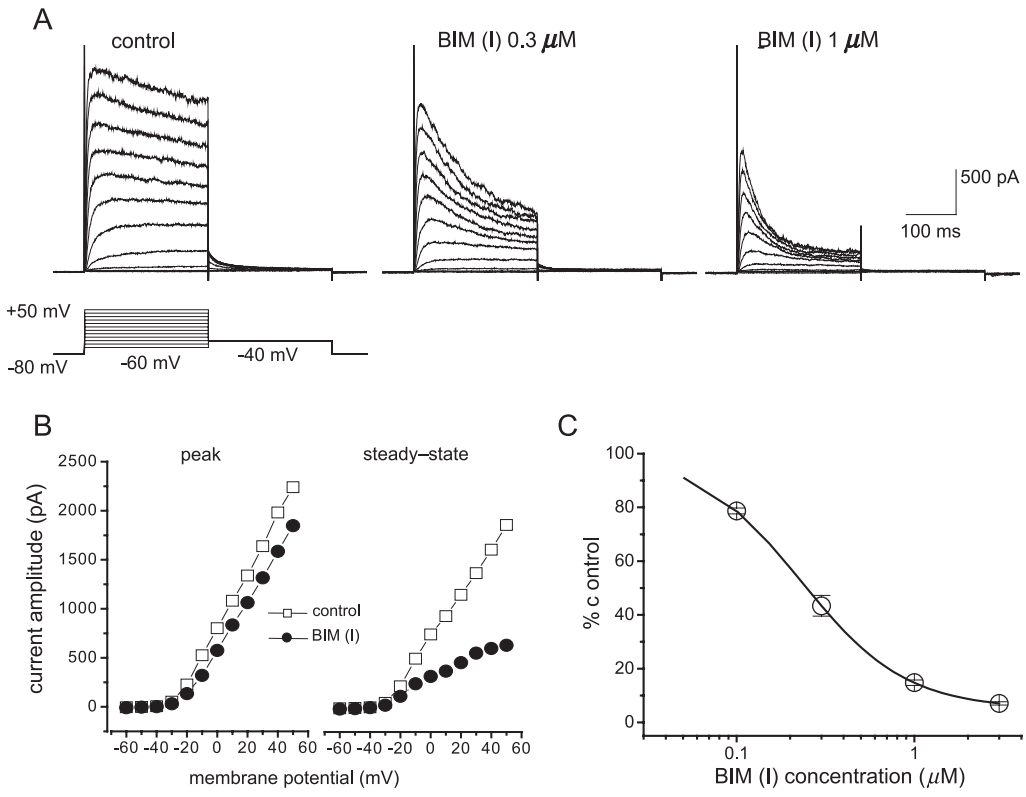

Fig. 1. Effects of bisindolylmaleimide (I) (BIM (I)) on the voltage-dependent K^+ (Kv) currents in rat mesenteric arterial smooth muscle cells. (A) Superimposed current traces were elicited by 250-ms depolarizing pulses between − 60 and + 50 mV from holding potential of − 80 mV in the absence and presence of bisindolylmaleimide (I). Tail currents were recorded with a repolarization to − 40 mV. The shape of voltage pulses is shown as figure inset. (B) Current–voltage (I–V) relationships of the peak and quasi steady-state Kv currents in the absence and presence of bisindolylmaleimide (I). (C) The average concentration-dependence of the Kv current inhibition by bisindolylmaleimide (I) (n = 8). The drug-induced inhibition of the Kv currents was measured at the end of 250-ms depolarizing pulse of + 40 mV and normalized by control current amplitude. The smooth line represents the best fitting with Hill equation.

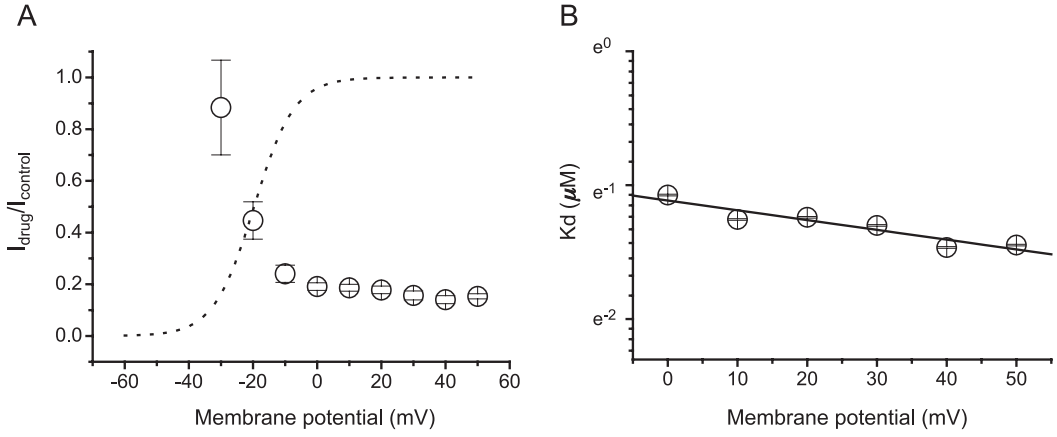

Fig. 2. Voltage-dependence of bisindolylmaleimide (I)-inhibition of the Kv currents. (A) Normalized inhibition shown as relative current amplitude ($I_{BIM\ (I)}$/$I_{control}$) at each potential (n = 5). The dotted line represents the activation curve of the Kv currents under control condition, which was conventionally obtained from deactivating tail current amplitude at − 40 mV with the following Boltzmann equation:

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

where k represents the slope factor, V the test potential and $V_{1/2}$ the voltage at which the conductance was half-maximal. (B) Semilogarithmic plot of the concentration of bisindolylmaleimide (I) for half-maximum inhibition (K_d) against the membrane potential. The K_d values for each membrane potential were obtained from concentration–response relations. The linear line represents the least-square fitting by Woodhull equation (see the text for detail).

solution and 10 mM BAPTA was added to the pipette solution. ATP-sensitive K^+ (K_{ATP}) channels were also inhibited by 5 mM MgATP in the pipette solution. Under this experimental condition, step depolarizations above − 40 mV from the holding potential of − 80 mV elicited typical vascular smooth muscle Kv currents. The representative current traces are shown in left panel of Fig. 1A. The currents were rapidly activated and then slowly and partially inactivated during depolarizations above + 10 mV, whereas the currents elicited by mild depolarizations between − 30 and 0 mV showed little inactivation.

The effects of bisindolylmaleimide (I) on the Kv currents are illustrated in the middle and right panels of Fig. 1A. In the presence bisindolylmaleimide (I), the current decay during the step depolarization was much faster than that observed without the drug, and the quasi-steady-state currents measured at the end of 250-ms depolarizing pulse decreased in a concentration-dependent manner. The peak current amplitude was relatively little affected. The washout of the drug usually restored the current to more than 80% of control values (data not shown), indicating that the effect of bisindolylmaleimide (I) is reversible. Fig. 1B shows the peak and steady-state current–voltage (I–V) relationships of the Kv currents in the absence and presence of bisindolylmaleimide (I). The steady-state I–V relationship indicated that the bisindolylmaleimide (I)-Kv current inhibition was voltage-dependent, the blockade increasing as depolarization increased. Fig. 1C summarizes the concentration-dependence of the Kv current inhibition. The bisindolylmaleimide (I)-induced inhibition of the Kv currents was measured at the end of 250-ms depolarizing pulse of + 40 mV and was normalized by current amplitude in the absence of drug. A non-linear least-square fit of the Hill equation to the concentration-dependence data yielded an apparent K_d value and a Hill coefficient of 0.23 ± 0.001 μM and 1.48 ± 0.004 (n = 8), respectively.

3.2. Voltage-dependence of bisindolylmaleimide (I)-Kv current inhibition

Fig. 2 shows the voltage-dependent inhibition of bisindolylmaleimide (I) of the Kv currents. To quantify the effects of the voltage on the inhibition, the relative current ($I_{drug}/I_{control}$) was plotted as a function of membrane potential (Fig. 2A). The dotted line shows the activation curve of the Kv current from control experiments (n = 10). The current started to activate above − 40 mV and the full activation of the channel conductance was achieved by depolarization above 0 mV. The midpoint and slope conductance from Boltzmann equation yielded values of − 19.5 ± 0.9 and 6.1 ± 0.7 mV, respectively. In the presence of bisindolylmaleimide (I) (1 μM), the blockade increased steeply between − 30 and 0 mV, which corresponds to the voltage range of the opening (activation) of channels. An additional low degree of inhibition with a shallow voltage-dependence was detected in the voltage range between 0 and + 50 mV despite the Kv currents are fully activated over this voltage (Fig. 2A). We further investigated the voltage-dependence above 0 mV. Fig. 2B shows the K_d values between membrane potential of 0 and + 50 mV. The K_d values for the respective membrane potentials were obtained from respective concentration–response curve like in Fig. 1C. Under assumption that bisindolylmaleimide (I) interacts intracellularly with the Kv channels, we fitted the data using following Woodhull equation:

$$K_d(E) = K_d(0)\exp(z\delta EF/RT)$$

The slope was 136.8 mV for e-fold change of K_d values, from which δ value was calculated to be ~ 0.19.

3.3. Time-dependence of bisindolylmaleimide (I)-Kv current inhibition

As shown in Fig. 1, the inhibition of the Kv current of rat mesenteric arterial smooth muscle cells was mainly

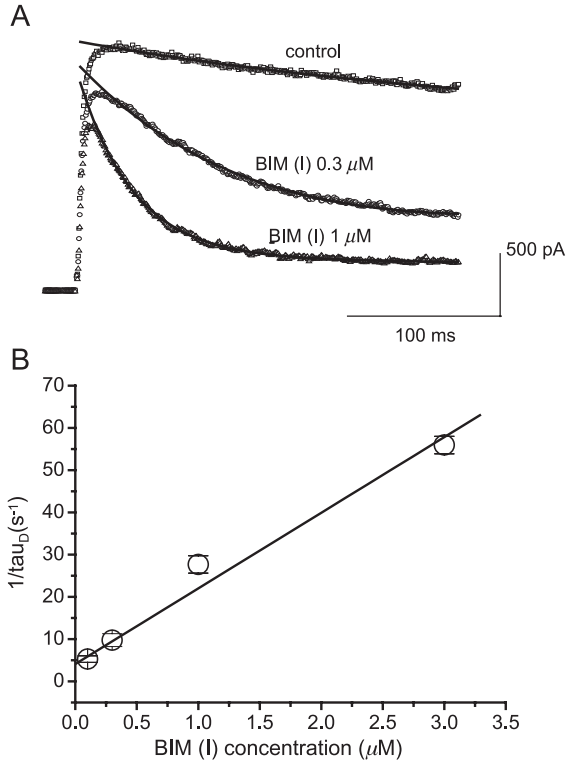

Fig. 3. The kinetics of the Kv current inhibition by bisindolylmaleimide (I). (A) Currents were elicited by depolarization to + 40 mV from holding potential of − 80 mV in the absence and presence of various concentrations of bisindolylmaleimide (I). The time constants of apparent current decay were obtained by single (control) or double exponential fit (under presence of bisindolylmaleimide (I)). The smooth lines represent the best fit. The reciprocals of τ_D at + 40 mV were plotted against the bisindolylmaleimide (I) concentration. The solid line represents the least-square fit of the data to the relation $1/\tau_D = k_{+1}[D] + k_{-1}$. An apparent association rate constant (k_{+1}) of 17.9 ± 1.6 μM^{-1} s^{-1} and dissociation rate constant (k_{-1}) of 4.1 ± 1.5 s^{-1} were obtained from the slope and intercept value of the fitted line (n = 6).

by the concentration-dependent acceleration of current decay. The decay time courses of Kv currents in the absence and presence of bisindolylmaleimide (I) are illustrated in Fig. 3. The control Kv currents slowly and partly decayed with time constant of 282.6 ± 50.3 ms ($n=6$) at +40 mV due to its intrinsic inactivation (Nelson and Quayle, 1995; Archer et al., 1998). We, therefore, fitted the currents in the presence of bisindolylmaleimide (I) with double exponentials assuming that other faster component is time-dependent block by bisindolylmaleimide (I). In Fig. 3B, the reciprocal of the time constant (τ_D) for Kv current inhibition at +40 mV was plotted as a function of bisindolylmaleimide (I) concentration. Apparent association rate constant (k_{+1}) of 17.9 ± 1.6 μM^{-1} s^{-1} and dissociation rate constant (k_{-1}) of 4.1 ± 1.5 s^{-1} ($n=6$) was obtained from the least-square fitting of the above data according to the following equation:

$$1/\tau_D = k_{+1}[D] + k_{-1}$$

From the following relation, the theoretical K_d value was calculated to be ~0.23 μM.

$$K_d = k_{-1}/k_{+1}$$

The derived K_d of 0.23 μM was well corresponded to the apparent K_d of 0.23 μM, which was obtained from the concentration–response curve shown in Fig. 1C.

3.4. Effects of bisindolylmaleimide (I) on the steady-state activation and inactivation of the Kv currents

The steady-state activation and inactivation kinetics of Kv currents in the absence and presence of bisindolylmaleimide (I) were investigated. Fig. 4A shows the normalized $I-V$ relationships of the peak Kv currents in the absence and presence of bisindolylmaleimide (I) ($n=6$). They were almost completely overlapped to each other, indicating that bisindolylmaleimide (I) did not affect the steady-state activation kinetics of the Kv currents. The steady-state inactivation of the Kv currents was investigated using a conventional double-pulse protocol, of which shape is shown in Fig. 4B as inset. The amplitude of the current peaks measured at +40 mV after 7-s prepulse was normalized to the maximum amplitude. The values were plotted against the prepulse potentials, and fitted to the Boltzmann equation. The potential ($V_{1/2}$) of the half-inactivation point and slope value (k) of the steady state inactivation curves were -25.7 ± 0.5 and 6.0 ± 0.4 mV for the control and -29.5 ± 0.5 and 6.3 ± 0.4 mV for bisindolylmaleimide (I), respectively ($n=6$). The slight leftward shift of $V_{1/2}$ and small change in k by bisindolylmaleimide (I) were not statistically significant. The results suggest that bisindolylmaleimide (I) is unlikely to interact with the inactivated state of the Kv channels of rat mesenteric arterial smooth muscle cells.

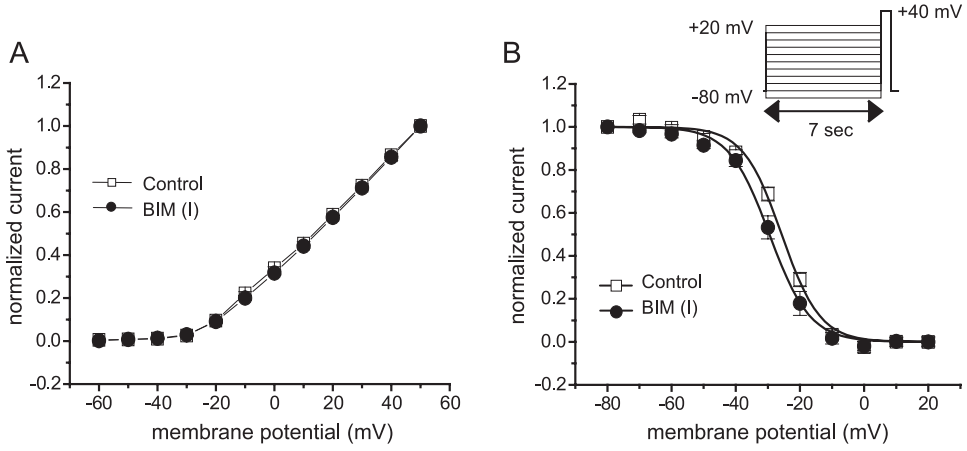

Fig. 4. Effect of bisindolylmaleimide (I) on the steady-state activation and inactivation of the Kv currents. (A) The peak $I-V$ relationships, which were obtained like in Fig. 1B, were normalized by the current value at +50 mV under control condition and during 0.3 μM bisindolylmaleimide (I) application. (B) The steady-state inactivation curves (solid line) of Kv channels under control condition and during 0.3 μM bisindolylmaleimide (I) application were obtained using a typical double-pulse protocol, of which shape is shown as figure inset, with the following Boltzmann equation:

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

where V is the preconditioning potential, and $V_{1/2}$ and k represent the potential corresponding to the half-inactivation point and slope factor, respectively. $n=6$ for each (A) and (B). Error bar is not visible when it is smaller than the symbol size.

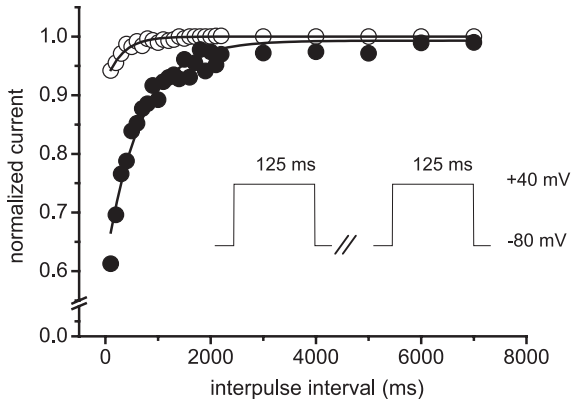

Fig. 5. Time course of the recovery from bisindolylmaleimide (I)-block of the Kv currents. To measure the degree of recovery, a double-pulse protocol was applied as shown in figure inset: A 125-ms conditioning prepulse from −80 to +40 mV was followed by a test pulse of the same duration and amplitude of voltage with various intervals (from 100 ms to 7 s). The peak amplitudes of the currents elicited by the test pulses were plotted as a function of the pulse intervals after being normalized by the peak amplitude of the current elicited by the corresponding prepulses (mean of five experiments, the error flag was omitted for clarity). The solid line represents the fitting of the plotted data to a single exponential function. The time constant of recovery under control condition was 275.0 ± 58.3 ms ($n=5$) and the recovery time constant in the presence of bisindolylmaleimide (I) was 577.2 ± 52.7 ms ($n=5$, $P<0.01$).

3.5. Recovery kinetics of the Kv current from bisindolylmaleimide (I)-block

Recovery from bisindolylmaleimide (I)-induced block was measured by using a double pulse protocol, which was shown as inset in Fig. 5. The interpulse interval between two pulses was progressively increased from 100 ms to 7 s. Fig. 5 shows the recovery kinetics of the Kv currents from bisindolylmaleimide (I)-block. The data were well fitted by a single exponential with recovery time constants of 275.0 ± 58.3 ms ($n=5$) under control condition and 577.2 ± 52.7 ms ($n=5$, $P<0.01$) in the presence of 0.3 μM bisindolylmaleimide (I). The slower recovery time constant of ~ 577 ms implicates the use-dependence of bisindolylmaleimide (I)-block (Fig. 6). The time constant of ~ 580 ms also implicates that the complete recovery of the Kv current from the use-dependent bisindolylmaleimide (I)-block needs at least ~ 2.5 s, which is four times of the recovery time constant. In agreement with this prediction, application of 125-ms pulses of +40 mV at 0.25 Hz did not produce any use-dependent block of Kv current by bisindolylmaleimide (I) (data not shown). It is of interest to note that the time constant of recovery from block at −80 mV is ~ 577, since it implicates the off rate constant of ~ 1.7 s^{-}, which is relatively

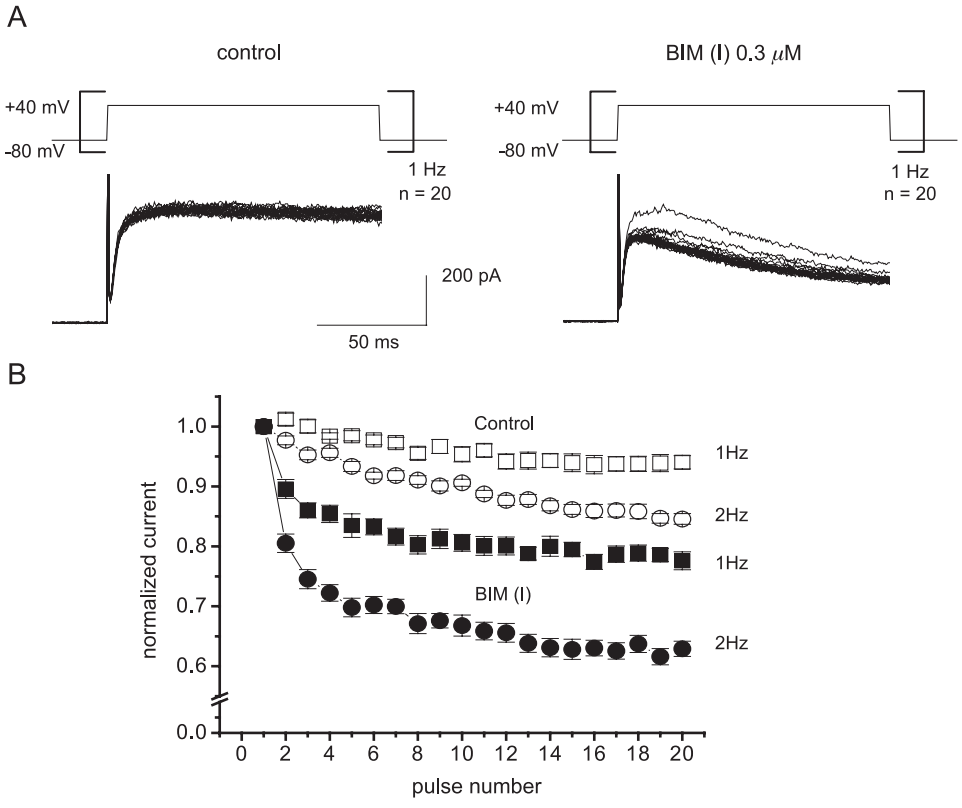

Fig. 6. Frequency-dependent inhibition of the Kv current by bisindolylmaleimide (I). (A) Twenty repetitive 125-ms depolarizing pulses of +40 mV from a holding potential of −80 mV, of which shape is shown as figure inset, were applied at a frequency of 1 Hz, and the corresponding current traces are superimposed. (B) The peak amplitudes of the Kv currents obtained by two different frequencies of the pulse train (1 and 2 Hz) under control conditions (open symbols) and in the presence of 0.3 μM bisindolylmaleimide (I) (filled symbols) were plotted against the pulse numbers after normalized by the peak amplitude of the Kv current elicited by the first number of pulse ($n=10$).

similar to the off rate constant of 4.1 s^{-} at +40 mV (Fig. 3). This suggest that the drug is not trapped in the channel on hyperpolarization, because in that case we would expect much different values between potentials where the channel is open (+40 mV) or closed (−80 mV).

3.6. Use-dependence of bisindolylmaleimide (I) action on the Kv currents

Fig. 6 shows the use-dependence of bisindolylmaleimide (I)-block of the Kv currents. Twenty repetitive 125-ms depolarizing pulses of +40 mV from a holding potential of −80 mV were applied at two different frequencies, 1 and 2 Hz. The use-dependence was tested at least after 5-min exposure with bisindolylmaleimide (I), which was sufficient time for achieving steady level of inhibition. Panel A shows the superimposed current traces under control and in the presence of 0.3 μM bisindolylmaleimide (I). Note little difference between the peak amplitudes of the first currents in the absence and presence of bisindolylmaleimide (I), but marked reduction of peak amplitudes of second current in the presence of bisindolylmaleimide (I). Under control conditions, the peak amplitude of the Kv current elicited by the last 20th depolarizing pulse was 94.0 ± 1.1% (at a frequency of 1 Hz) and 84.6 ± 0.9% (at a frequency of 2 Hz) of the first current amplitude. In the presence of 0.3 μM bisindolylmaleimide (I), the peak amplitude of the Kv current amplitude progressively decreased and the 20th current amplitude reached 77.6 ± 1.5% (at a frequency of 1 Hz) and 62.9 ± 1.3% (at a frequency of 2 Hz, $n = 10$) of the amplitude of first current. These data indicate that the blocking-action of the bisindolylmaleimide (I) is frequency-dependent.

3.7. Effects of bisindolylmaleimide (V) on the Kv currents

Fig. 7 shows the effect of bisindolylmaleimide (V), an inactive analogue of bisindolylmaleimide (I), on the Kv currents. Similarly to bisindolylmaleimide (I), bisindolylmaleimide (V) inhibited the Kv currents reversibly and concentration-dependently, especially affecting the steady-state level by accelerating current decay (Fig. 7A). Fig. 7B illustrates the peak and steady-state I–V relationships of the Kv current in the absence and presence of bisindolylmaleimide (V). Fig. 7C summarizes the concentration-dependence of the Kv current inhibition by bisindolylmaleimide (V). The bisindolylmaleimide (V)-induced inhibition of the Kv currents was measured at the end of 250-ms depolarizing pulse of +40 mV and normalized by current amplitude in the absence of the drug. A non-linear least-square fit of the Hill equation to the concentration-dependence data yielded

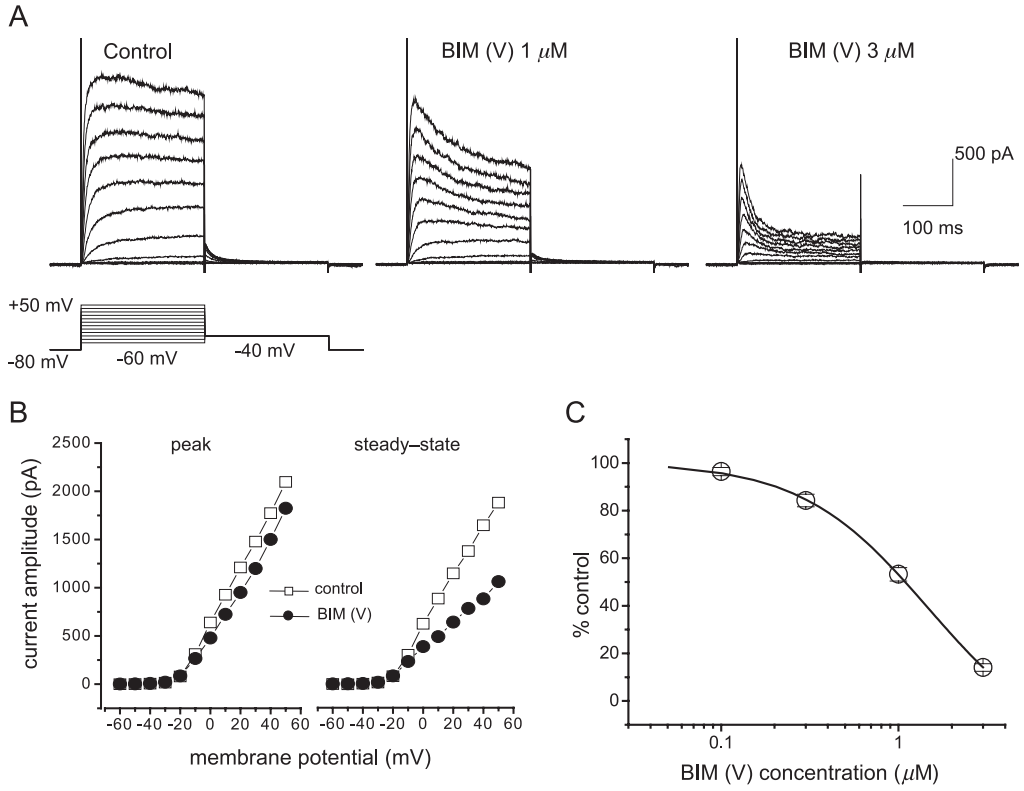

Fig. 7. Effects of bisindolylmaleimide (V) on the voltage-dependent K^{+} (Kv) currents in rat mesenteric arterial smooth muscle cells. (A) Superimposed Kv current traces in the absence and presence of bisindolylmaleimide (V). The shape of voltage pulses is shown as figure inset. (B) *I–V* relationships of the peak and quasi steady-state Kv currents in the absence and presence of bisindolylmaleimide (V). (C) The average concentration-dependence of the Kv current inhibition by bisindolylmaleimide (V) ($n = 6$). The drug-induced inhibition of the Kv currents was measured at the end of 250-ms depolarizing pulse of +40 mV and normalized by control current amplitude. The smooth line represents the best fitting with Hill equation.

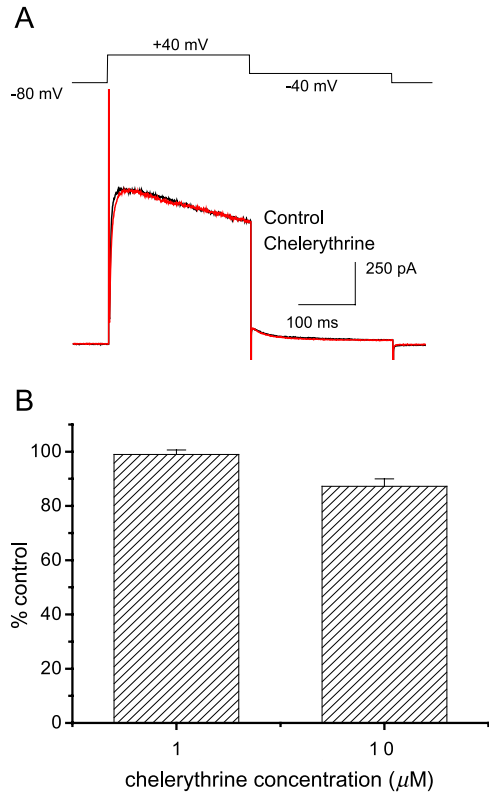

Fig. 8. The effect of chelerythrine on the Kv currents. Whole-cell currents were elicited with voltage pulse, of which shape is illustrated as figure inset. (A) Control current and current during chelerythrine (1 μM) application. (B) Summary for the effect of chelerythrine on the Kv currents ($n=7$). The drug-induced effect of the Kv currents was measured at the end of 250-ms depolarizing pulse of +40 mV and normalized by current amplitude in the absence of the drug.

an apparent K_d value and a Hill coefficient of 1.47 ± 0.19 μM and 1.21 ± 0.087 ($n=6$), respectively.

3.8. The effects of other PKC inhibitor chelerythrine on the Kv currents

In order to confirm the inhibition by bisindolylmaleimide (I) of the Kv currents of rat mesenteric arterial smooth muscle cells was indeed PKC-independent, we tested other PKC inhibitor, chelerythrine, which is structurally different from bisindolylmaleimide. Fig. 8 shows the effects of chelerythrine on the Kv currents. In contrast to the effect of bisindolylmaleimide (I) and bisindolylmaleimide (V), chelerythrine failed to inhibit the Kv currents. These results support the idea that inhibition of the Kv currents of rat mesenteric arterial smooth muscle cells by bisindolylmaleimide (I) was not through PKC inhibition but a direct effect on Kv channels.

4. Discussion

In the present study, we have showed the effect of bisindolylmaleimide (I) on the Kv current of rat mesenteric arterial smooth muscle cells. The results provided evidence for state-, voltage-, time- and use-dependent inhibition of the Kv currents in rat mesenteric arterial smooth muscle cells by bisindolylmaleimide (I). Bisindolylmaleimide (I) is well known as a specific PKC inhibitor ($IC_{50}=20$ nM). However, effects of bisindolylmaleimide (I) on targets other than PKC have also been identified recently. bisindolylmaleimide (I) inhibited voltage-dependent Na^+ channels (Lingameneni et al., 2000), acetylcholine-activated K^+ current in atrial myocytes from mice (Cho et al., 2001), and mouse 5-HT_3 receptors (Coultrap et al., 1999) with a potency comparable to that for inhibition of PKC. Inhibition of Kv currents of vascular smooth muscle cells must be added to the growing list of additional effects of the "selective" PKC inhibitor, bisindolylmaleimide (I).

4.1. Bisindolylmaleimide (I) directly interact with the Kv channels of rat mesenteric arterial smooth muscle cells independently of PKC inhibition

The inhibition seem not to be through blockade of PKC but to be directly on the Kv channels due to following reasons: (1) bisindolylmaleimide (V), of which reference half-inhibition value for PKC inhibition is >100 μM and thus used as an inactive analogue of bisindolylmaleimide (I), inhibited the Kv currents of rat MASCMs with comparable potency as bisindolylmaleimide (I), with a half-inhibition value of 1.47 μM. (2) The half-inhibition value of bisindolylmaleimide (I) on the Kv currents of rat mesenteric arterial smooth muscle cells was 0.23 μM in the present study. This value was much lager than the reference half-inhibition value of 0.01 μM for PKC inhibition, and significantly smaller than the half-inhibition reference value of 2 μM for PKA inhibition, which indicates that the inhibitory effect on the Kv current is due to the inhibition of neither PKC nor PKA. (3) Other PKC inhibitor chelerythrine, which is structurally different from bisindolylmaleimide, had no effect on the Kv currents. Moreover, (4) the activation of PKC rather than the inhibition of this enzyme has been reported to inhibit the Kv currents in vascular smooth muscle (Clement-Chomienne et al., 1996; Shimoda et al., 2001; Yeon et al., 2001). These facts strongly suggest that bisindolylmaleimide (I) directly interacts with the Kv channels of rat mesenteric arterial smooth muscle cells independently of PKC.

4.2. The mechanism by which bisindolylmaleimide blocks the Kv currents of rat mesenteric arterial smooth muscle cells

The inhibition of Kv currents by bisindolylmaleimide (I) in the present study is characterized by concentration-dependent acceleration of the current decay, which is normally seen with open channel blockers where the drug

binding rate is faster for the open channel than the inactivated channel (Colquhuon and Hawkes, 1983; Valenzuela et al., 1996; Delpon et al., 1997; Choi et al., 2000). The characteristics of the bisindolylmaleimide (I)-induced block of the Kv currents suggest that bisindolylmaleimide (I) blocks the open state of the channels. Evidence for this mechanism includes the following results. First, bisindolylmaleimide accelerated the rate of Kv current decay with little effect on the time course of activation and peak current amplitude. Second, the block of Kv currents by bisindolylmaleimide (I) was voltage-dependent. The blockade increased steeply in the voltage range of channel activation (between −30 and 0 mV), suggesting that the drug-channel interaction require channel activation (opening). Over the voltage range of full activation of Kv currents (between 0 and +50 mV), bisindolylmaleimide (I) displayed further slight voltage-dependence ($\delta = \sim 0.19$). bisindolylmaleimide (I) is a weak base with a $pK_a = 8.52$. Therefore, at the intracellular pH of 7.3 (pH of the pipette solution), bisindolylmaleimide (I) is mainly protonated or positive charged. If a drug has a high affinity for activated and/or open channels, the voltage-dependence of channel activation is superimposed on the intrinsic voltage-dependence of drug binding because channel activation makes the receptor available. The total voltage-dependence of block is then composed of a steep phase that reflects channel activation and a shallower phase that reflects the additional effect of the electrical field on the charged drug (see Fig. 2). Therefore, the δ value of 0.19 observed in the presence of bisindolylmaleimide (I) for the voltage-dependence of the apparent K_d can be interpreted to indicate that the single positive charge of bisindolylmaleimide (I) senses 19% of the membrane electric field. The mechanism for the block of Kv currents by bisindolylmaleimide (I) in the present study is similar to that for the block of rKv1.5 by bisindolylmaleimide (I), which was previously reported by Choi et al. (2000). Bisindolylmaleimide (I) inhibited the rKv1.5 in CHO cells also in a voltage-, state-, time- and use-dependent manner. The half inhibition value of the bisindolylmaleimide (I) on the Kv current of rat mesenteric arterial smooth muscle cells in the present study was 0.23 μM and it was 0.38 μM for rKv 1.5 (Choi et al., 2000). The half inhibition values of the bisindolylmaleimide (V) on the Kv current in the present study and on the rKv1.5 (Choi et al., 2000) were also corresponded: 1.47 and 1.70 μM, respectively. These results suggest that bisindolylmaleimide inhibits the rKv1.5 and the Kv channels of the rat mesenteric arterial smooth muscle cells with very similar mechanism, i.e., open channel block.

In summary, the present study demonstrated the direct block of the Kv currents of rat mesenteric arterial smooth muscle cells by bisindolylmaleimide (I). The result warns that much caution is required when using PKC inhibitor, bisindolylmaleimide (I), for the study of PKC function in vascular smooth muscle.

Acknowledgements

This work was supported by the Faculty Fund of Konkuk University in 2001.

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