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Chelerythrine and bisindolylmaleimide I prolong cardiac action potentials by protein kinase C-independent mechanism

Sari Voutilainen-Myllylä, Pasi Tavi, Matti Weckström*

Department of Physical Sciences, Division of Biophysics, University of Oulu, P.O. Box 3000, FIN-90014 Oulu yliopisto, Finland
Department of Physiology and Biocenter Oulu, P.O. Box 3000, FIN-90014 Oulu yliopisto, Finland

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Abstract

Effects of chelerythrine and bisindolylmaleimide I on action potential duration and on voltage-activated K^+ and Ca^{2+} currents in rat ventricular myocytes were studied using perforated patch-clamp technique. The action potentials were markedly prolonged after application of 20 μM chelerythrine or 100 nM bisindolylmaleimide I. Chelerythrine and bisindolylmaleimide I reduced the amplitude of sustained current ($I_{K,sus}$) significantly. Transient K^+ current (I_{to}) was inhibited only by chelerythrine. Ca^{2+} current was reduced only with highest chelerythrine concentration (50 μM). Application of chelerythrine and bisindolylmaleimide I inhibited outward K^+ currents significantly also in ruptured patch-clamp configuration. Bisindolylmaleimide V, an inactive analogue of bisindolylmaleimide I, decreased $I_{K,sus}$ substantially. However, I_{to} and $I_{K,sus}$ were not affected by calphostin C. Direct protein kinase C activators resulted in decrease of outward K^+ currents. Chelerythrine blocked I_{to} in a use-dependent manner and the block did not recover during a 4-min washout. $I_{K,sus}$ was not blocked by this mechanism by either inhibitor. We conclude that chelerythrine and bisindolylmaleimide I inhibit outward K^+ currents independently of protein kinase C inhibition.

Keywords: Bisindolylmaleimide I; Ca^{2+} current; Calphostin; Clelerythrine; K^+ current; Protein kinase C

1. Introduction

Protein kinase C plays a central role in cellular signal transduction. One of the most straightforward methods to study the role of protein kinase C in cellular processes is to inhibit the enzymatic activity of the kinase in intact cells by a specific inhibitor. Several cell permeable and potent protein kinase C inhibitors, like chelerythrine and bisindolylmaleimide I, have been widely used in vivo and in vitro experiments. Both chelerythrine (Herbert et al., 1990) and bisindolylmaleimide I (Toullec et al., 1991) inhibit protein kinase C by interacting with the catalytic domain of protein kinase C. However, especially during the last few years, the specificity of these substances has become questionable. Chelerythrine has been reported to have nonspecific actions, like interaction with cyclic nucleotide phosphodiesterases (Eckly-Michel et al., 1997), inhibition of phospholipase D (Guillemain and Rossignol, 1995) and inhibition of acetylcholine-induced currents (Shi and Wang, 1999). Chelerythrine has also been reported not to inhibit protein kinase C (Lee et al., 1998). Recently, both chelerythrine and bisindolylmaleimide I have been shown to inhibit acetylcholine-sensitive K^+ current independently of protein kinase C inhibition (Cho et al., 2001). Bisindolylmaleimide I has also been demonstrated to be a potent antagonist of 5-hydroxytryptamine at 5-HT_3 receptor (Coultrap et al., 1999). In addition, bisindolylmaleimide I (Choi et al., 2000) and structurally similar staurosporine (Choi et al., 1999) have been reported to have direct ion channel effects on Kv 1.5 and Kv 1.3 channels, respectively. Due to nonspecific effects, the usefulness of these substances as specific protein kinase C inhibitors may be limited.

The possibility for nonspecific effects of protein kinase C inhibitors on cardiac ion channels have not been studied. In cardiac tissue, modulation of ion channel function leads to dramatic changes in action potential duration. This will further influence, for example, the contraction force of the heart. The present study was designed to evaluate the effects of chelerythrine and bisindolylmaleimide I, two widely used protein kinase C inhibitors, on cardiac ionic currents, which mainly define the speed of repolarization: (1) the fast-

* Corresponding author. Department of Physical Sciences, Division of Biophysics, University of Oulu, P.O. Box 3000, FIN-90014 Oulu yliopisto, Finland. Tel.: +358-8-537-5317; fax: +358-8-537-5320.
E-mail address: Matti.Weckstrom@oulu.fi (M. Weckström).

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activating and inactivating transient K^+ current (I_{to}), (2) the sustained K^+ current ($I_{K,sus}$), (3) the background inward rectifier K^+ current (I_{K1}) and (4) Ca^+ current (I_{Ca}). We also examined the role of protein kinase C inhibition in the observed effects of chelerythrine and bisindolylmaleimide I.

2. Materials and methods

2.1. Chemicals

NaCl, KCl, $MgCl_2$ and glucose were obtained from J.T. Baker (Deventer, The Netherlands), $CaCl_2$, KH_2PO_4 and ATP-Na_2 from Merck (Darmstadt, Germany) and $MgSO_4$ from Reidel-de Haën (Seelze, Germany). Taurine, HEPES, CsCl, $CdCl_2$ and Amphotericin B were from Sigma (St. Louis, MO, USA). Tetrodotoxin was purchased from Molecular Probes (Leiden, The Netherlands). Chelerythrine, bisindolylmaleimide I (GF109203X), bisindolylmaleimide V, calphostin C, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) and 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) were from Calbiochem (La Jolla, USA). Collagenase (CLS2) was obtained from Worthington Biochemical (NJ, USA) and EGTA from Sigma-Aldrich (Steinheim, Germany).

2.2. Isolation of single myocytes

Single myocytes from left and right ventricles were isolated by an enzymatic dissociation method described previously (Szokodi et al., 2002). Male Sprague–Dawley rats weighing 250–300 g were used. Isolated cells were stored at room temperature (+21–24 °C). Data collection was made 1–8 h after dissociation.

2.3. Patch-clamp recordings

The whole-cell membrane currents and voltages were recorded by the Amphotericin B perforated patch-clamp method (Rae et al., 1991) to avoid dialysis of cytosolic components and concomitant changes in ionic currents. In some experiments, the ruptured patch-clamp configuration was used. Electrodes were pulled from 1.2 mm O.D. borosilicate glass (Clark Electromedical, England) to have a resistance of 1.8–2.5 MΩ. Only recordings from cells with low access resistance (<20 MΩ) and high seal resistance (>1 GΩ) were included in the present study. Stimulation, data acquisition and analysis were controlled with Digidata 1200 (Axon Instruments, USA) and pClamp 7.0 (Axon Instruments) software. The recording system consisted of an Axopatch-1D CV4-1/100 (Axon Instruments) patch-clamp amplifier. Signals were filtered at 2 kHz. After the formation of the "Giga-seal" between the cell and the pipette, pipette capacitance was fully compensated electronically. Series resistance (R_s) was compensated ~ 60–80% in order to minimize the voltage error resulting from voltage drop over uncompensated resistance. Perforated patch-clamp experiments were performed at +29–30 °C and ruptured patch-clamp experiments at 21–23 °C.

2.4. Recording solutions and drugs

For perfusion, the cells were placed in a small chamber on the stage of an inverted microscope. In current clamp experiments, the external solution contained (in mM) 145 NaCl, 5.4 KCl, 1 $MgCl_2$, 1.5 $CaCl_2$, 10 glucose, 10 HEPES, and pH was adjusted to 7.4 with NaOH. The solution in the recording pipette contained (in mM) 150 KCl, 10 NaCl, 10 HEPES, and pH was adjusted to 7.2 with KOH. Outward K^+ currents were measured in external solution containing (in mM) 140 NaCl, 4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES, and pH was adjusted to 7.4 with NaOH. Na^+, Ca^{2+} and inward rectifier (I_{K1}) currents were blocked by 30 μM tetrodotoxin, 0.3 mM $CdCl_2$ and 4 mM CsCl, respectively. To study I_{K1}, CsCl was replaced with equimolar concentration of NaCl. In perforated patch-clamp experiments, the pipette solution contained (in mM) 155 KCl and 10 HEPES, pH was adjusted to 7.2 with KOH, whereas in ruptured configuration, the pipette solution contained (in mM) 137 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 Na_2-ATP, 10 HEPES, 10 EGTA, and pH was adjusted to 7.2 with KOH. For Ca^{2+}, current recordings myocytes were superfused with solution containing (in mM) 145 NaCl, 4 CsCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES, and pH was adjusted to 7.4 with NaOH. Pipette solution contained (in mM) 155 CsCl, 10 HEPES, and pH was adjusted to 7.2 with CsOH. Na^+ currents were blocked by tetrodotoxin (30 μM). Amphotericin B was dissolved in dimethyl sulfoxide (DMSO) (5 mg/100 μl) and then added to pipette solution for a final concentration of 200 μg ml^{-1}.

In the present paper, we studied the effects of chelerythrine (1, 7, 20 and 50 μM) and bisindolylmaleimide I (1, 10, 100 and 1000 nM) on action potential duration and ionic currents. In addition, the effects of calphostin C (1, 10, 100 and 1000 nM) and bisindolylmaleimide V (1, 10 100 and 1000 nM), an inactive analog of bisindolylmaleimide I, on outward K^+ currents were studied. Also, the effects of protein kinase C activators, OAG (10 and 100 μM) and TPA (10 and 100 nM) on outward K^+ currents were tested but not studied in detail. All drugs were rapidly applied to the cell by local perfusion using a motor-driven solution exchange device (RSC-200, Bio-logic, Claix, France). In this case, the delay between the on command and drug arrival at the cell membrane was 100 ms. The drug concentration was raised gradually from lowest to highest concentration and cells were incubated in each drug concentration for 2 min before measurements.

2.5. Data recording procedures and analysis

In order to normalize the starting conditions in current clamp experiments, the membrane potential was set to −73 mV, which was near the lower end of the range of the resting

potential (− 68 to − 73 mV) under our recording conditions. Action potentials were elicited by current pulses (2 nA, 2.5 ms) injected through the patch electrode at 2 Hz. The action potential duration was measured from the 15th action potential of a train at 25% and 75% repolarization.

Outward K^+ currents were evoked by 400 ms voltage steps between − 40 and + 60 mV from the holding potential of − 60 mV applied at 0.1 Hz. The holding potential of − 60 mV was chosen since at that potential, the residual I_{Na}, which was not blocked by tetrodotoxin, was inactivated. I_{to} was evaluated as the difference between the peak outward current and the current at the end of a 400-ms voltage step. $I_{K,sus}$ was measured as the K^+ current at the end of the 400-ms voltage step. The amplitudes of I_{to} and $I_{K,sus}$ elicited at depolarizing voltage step to + 60 mV were used as the index to construct concentration–response relationships for the effect of chelerythrine, bisindolylmaleimide I and bisindolylmaleimide V. The remaining current in the presence of drug, expressed as the fraction of control, was plotted versus drug concentration. The points were fitted to the Hill equation:

$$F = F_{max} D^n / (IC_{50}^n + D^n)$$

where F is the current fraction of control, D is the drug (chelerythrine, bisindolylmaleimide I or bisindolylmaleimide V) concentration, IC_{50} is the concentration at which the response was half-maximally inhibited and n is the Hill coefficient. All fittings were made using Microcal Origin 5.0 software (OriginLab, USA).

A two-pulse protocol was used to assess the voltage dependence of steady-state inactivation of I_{to} and $I_{K,sus}$ during chelerythrine and bisindolylmaleimide I treatment. The prepulses were used to clamp the membrane potential to different voltages ranging from − 120 to 0 mV for 500 ms. Each prepulse was followed by a single test pulse, which depolarized the cell to + 60 mV for 400 ms. Voltage steps were applied at 0.05 Hz. Conductances at different prepulse potentials were estimated by determination of the relative conductance elicited by the test potential. That is,

$$G = I / (V_m - E_K)$$

where G is the relative conductance at test potential V_m, I is the current amplitude and E_K is the calculated K^+ reversal potential (− 96 mV in our experimental conditions). Conductances were plotted as a function of prepulse potential. Since a single Boltzmann function did not adequately describe the inactivation data, as previously reported by Himmel et al. (1999), fittings were made using a sum of two Boltzmann functions plus a residual component.

$$G = (a/\{1 + \exp[(V_p - V_{0.5,a})/k_a]\}) + (b/\{1 + \exp[(V_p - V_{0.5,b})/k_b]\}) + r$$

where a and b are the fractional amplitudes of the two functions and r is the residual component. V_p is the prepulse potential, $V_{0.5,a}$ and $V_{0.5,b}$ are the potentials at which the conductances are half-maximally inactivated, k_a and k_b are the slope factors of the respective current components.

To construct the steady-state activation–voltage relations for I_{to} and $I_{K,sus}$, currents were elicited by test pulses stepping from − 40 to + 80 mV for 400 ms in 10 mV increments at 0.1 Hz from the holding potential of − 60 mV. The steady-state activation curves for I_{to} and $I_{K,sus}$ were estimated by determination of the relative conductance as a function of test potential. Conductances were plotted as a function of test potential. The points were fitted to a Boltzmann equation.

$$G = 1/\{1 + \exp[(V_m - V_{0.5})/k]\}$$

where G is the conductance at test potential (V_m), $V_{0.5}$ is the potential at which the conductance is half-maximally activated and k is the slope factor.

I_{K1} was evoked from the holding potential of − 30 mV by 400 ms hyperpolarizing pulses between − 50 and − 130 mV in − 20 mV increments with a frequency of 0.1 Hz. I_{K1} was measured as the current amplitude at the end of the voltage step.

I_{Ca} was elicited from the holding potential of − 60 mV by 200 ms depolarizing pulses between − 40 and + 60 mV in + 20 mV increments with frequency of 0.1 Hz. I_{Ca} was measured as the peak inward current.

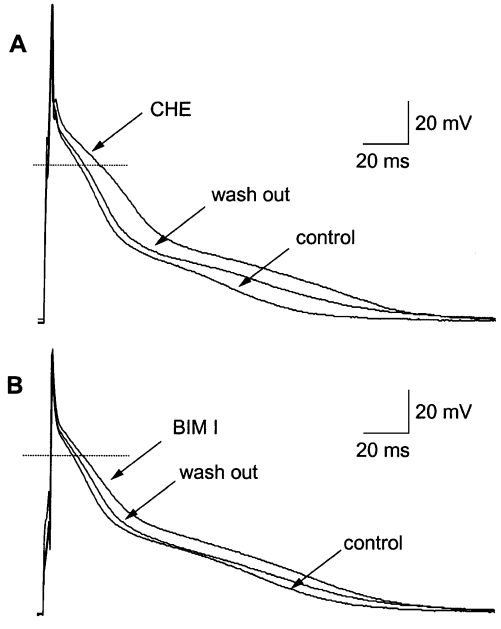

Fig. 1. An illustration of the effects of (A) chelerythrine (20 μM) and (B) bisindolylmaleimide I (100 nM) on action potentials in single ventricular myocytes. The resting potential was set to − 73 mV by current and action potentials were elicited by 2.5 ms current step with an amplitude of 2000 pA at 2 Hz. The 15th action potential of the train is shown in each case. The horizontal line indicates the level of 0 mV.

Use-dependency of drug (chelerythrine, bisindolylmaleimide I and bisindolylmaleimide V) action on I_{to} and $I_{K,sus}$ was studied by applying 15 repetitive 300 ms depolarizing pulses of +60 mV from the holding potential of −60 mV at 1 Hz. The amplitudes of I_{to} and $I_{K,sus}$ at every pulse were normalized by the current amplitude obtained at the first pulse and plotted against pulse number. These experiments were performed without substraction; otherwise, all currents were leak-substracted on-line using P/6 protocol.

2.6. Statistical analysis

All data are given as mean ± S.E.M. The effects of chelerythrine or bisindolylmaleimide I treatments on action potential duration were analysed using one-way analysis of variance with repeated measures (one-way RM ANOVA) followed by the Tukey test. Two-way analysis of variance with repeated measures (two-way RM ANOVA) followed by the Tukey test was used to analyse the effects of chelerythrine or bisindolylmaleimide I treatments on voltage–current relations. Otherwise, comparisons were made using the paired *t*-test. Differences at the 95% level ($P<0.05$) were considered as statistically significant. All statistical tests were made using SigmaStat 2.03 software (SPSS Science, USA).

3. Results

3.1. Effects of chelerythrine and bisindolylmaleimide I on action potential duration

The resting membrane potentials were −70.0 ± 1.0 and −71 ± 1.1 mV in chelerythrine- and bisindolylmaleimide I-treated groups, respectively ($n=6$). There was a marked increase in the duration of the action potential in the myocytes treated with chelerythrine and bisindolylmaleimide I, and the effect was partially reversible upon a 4-min washout of the drug, as illustrated in Fig. 1. The action potential in the presence of chelerythrine had a much slower rate of initial repolarization (Fig. 1A). The late phase of repolarization was also prolonged, although the effect was less pronounced than the effect on initial repolarization. On average, the application of 20 μM chelerythrine prolonged the action potential duration statistically significantly at 25% and 75% repolarization by 114% (from 2.8 ± 0.6 to 6.0 ± 1.3, $P<0.01$) and 126% (from 50.6 ± 12.1 to 114.2 ± 21.2, $P<0.01$), respectively. Fig. 1B shows an example of the effects of bisindolylmaleimide I (100 nM) on the action potential duration. Addition of bisindolylmaleimide I (100 nM, $n=6$) prolonged the action potential

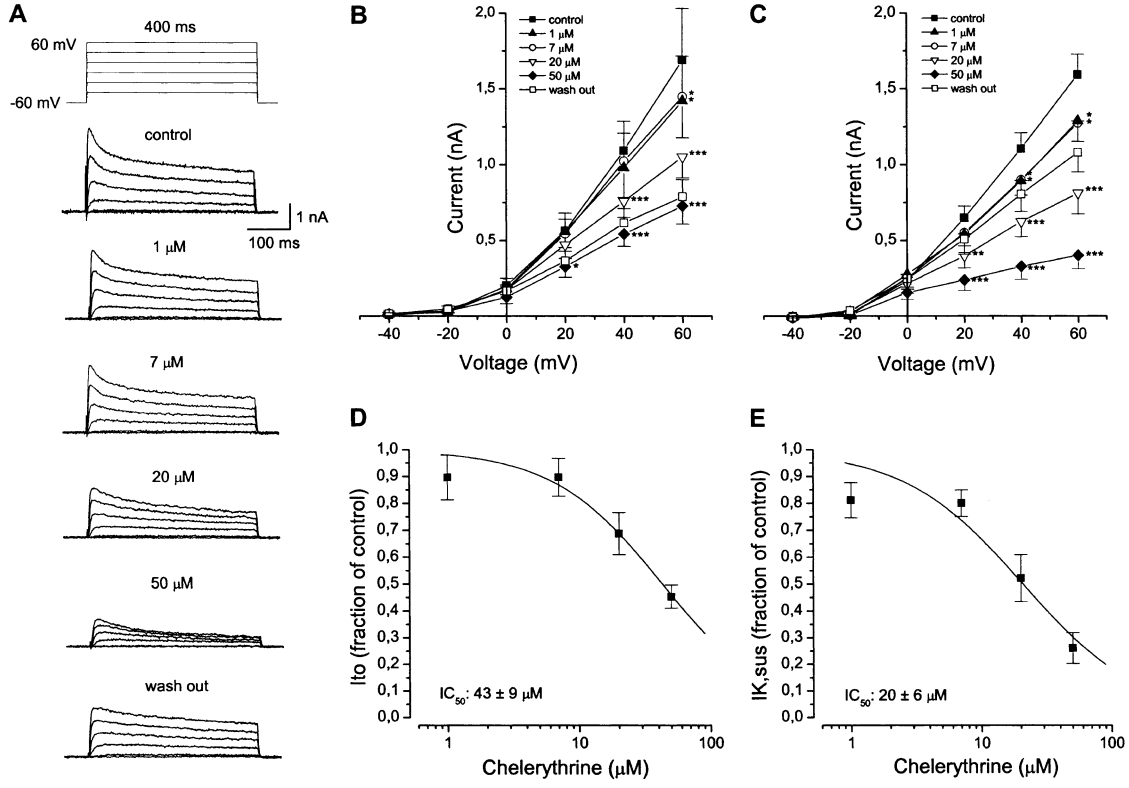

Fig. 2. Effects of chelerythrine (CHE) on outward K^+ currents. (A) Representative K^+ currents from a ventricular myocyte treated with chelerythrine (1, 7, 20 and 50 μM). Currents were elicited by test pulses stepping from −40 (lowest trace) to +60 mV (highest trace) in 20 mV increments, as indicated in the top panel. The holding potential was −60 mV. (B) Current–voltage relationship in the absence and presence of chelerythrine for I_{to} and (C) $I_{K,sus}$ (two-way RM ANOVA, $*P<0.05$, $**P<0.01$, $***P<0.001$). (D) Concentration–response curves for the inhibitory effect of chelerythrine on I_{to} and (E) $I_{K,sus}$. The amplitudes of the K^+ currents at depolarizing step to +60 mV were used to construct the concentration–response curves and the amplitudes are shown as the fraction of the control. The curves were fitted with the Hill equation (see Materials and methods).

duration at 25% and 75%, and repolarization on the average by 30% (from 2.3 ± 0.3 to 3.0 ± 0.6, $P>0.05$) and 25% (from 67.1 ± 13.8 to 84.1 ± 15.3, $P>0.05$), respectively.

3.2. Effects of chelerythrine and bisindolylmaleimide I on I_{to} and $I_{K,sus}$

Fig. 2A shows typical outward K^+ current traces with different concentrations of chelerythrine. Depolarizations more positive than −40 mV evoked outward K^+ currents and the amplitudes of total currents increased with more positive potentials. Chelerythrine decreased both I_{to} (Fig. 2B) and $I_{K,sus}$ (Fig. 2C) at depolarizing pulse potentials from +20 to +60 mV in a concentration-dependent manner. The $I_{K,sus}$ recovered partially during a 4-min washout, whereas the effect on I_{to} did not seem to recover during washout ($n=5$). The concentration for the half-maximal inhibition (IC_{50}) of I_{to} and $I_{K,sus}$ were 43 ± 9 and 20 ± 6 μM, respectively (Fig. 2D,E).

Typical current traces in the absence and presence of different concentrations of bisindolylmaleimide I are shown in Fig. 3A, and mean current–voltage relations for I_{to} and $I_{K,sus}$ are shown in Fig. 3B,C, respectively. $I_{K,sus}$ was reduced in response to bisindolylmaleimide I application ($n=5$). Bisindolylmaleimide I had no effect on I_{to} amplitude or time constant of current inactivation (control: 21.4 ± 0.8 ms; 100 mM bisindolylmaleimide I: 24.0 ± 0.9 ms; 1000 nM bisindolylmaleimide I: 20.0 ± 2.0 ms). The IC_{50} of bisindolylmaleimide I on $I_{K,sus}$ was 51 ± 32 nM (Fig. 3E) and the current recovered partially during a 4-min washout.

3.3. Effects of chelerythrine and bisindolylmaleimide on steady-state inactivation and activation of I_{to} and $I_{K,sus}$

Representative current traces before and after application of chelerythrine are shown in Fig. 4A (left traces). The amplitudes of I_{to} and $I_{K,sus}$ were reduced substantially when the prepulse progressively depolarized the cell membrane, suggesting that the number of available channels was decreased with membrane depolarization. In Fig. 4B,C (left curves) are plots of the data for I_{to} and $I_{K,sus}$ ($n=5$), respectively, obtained before and after 20 μM application. We used a sum of two Boltzmann functions to analyse the effects of protein kinase C inhibitors on parameters of steady-state inactivation (see Materials and methods). Chelerythrine treatment had an exceptional effect on I_{to} inactivation curve. As shown in Fig. 4B, the I_{to} component, which inactivates at voltages below −60 mV ($I_{to,a}$), is hardly detectable in control conditions, but after chelerythrine treatment, it becomes clearly visible. Application of 20 μM chelerythrine resulted in a statistically significant hyperpolarizing shift in the inactivation curve for $I_{to,a}$ by decreas-

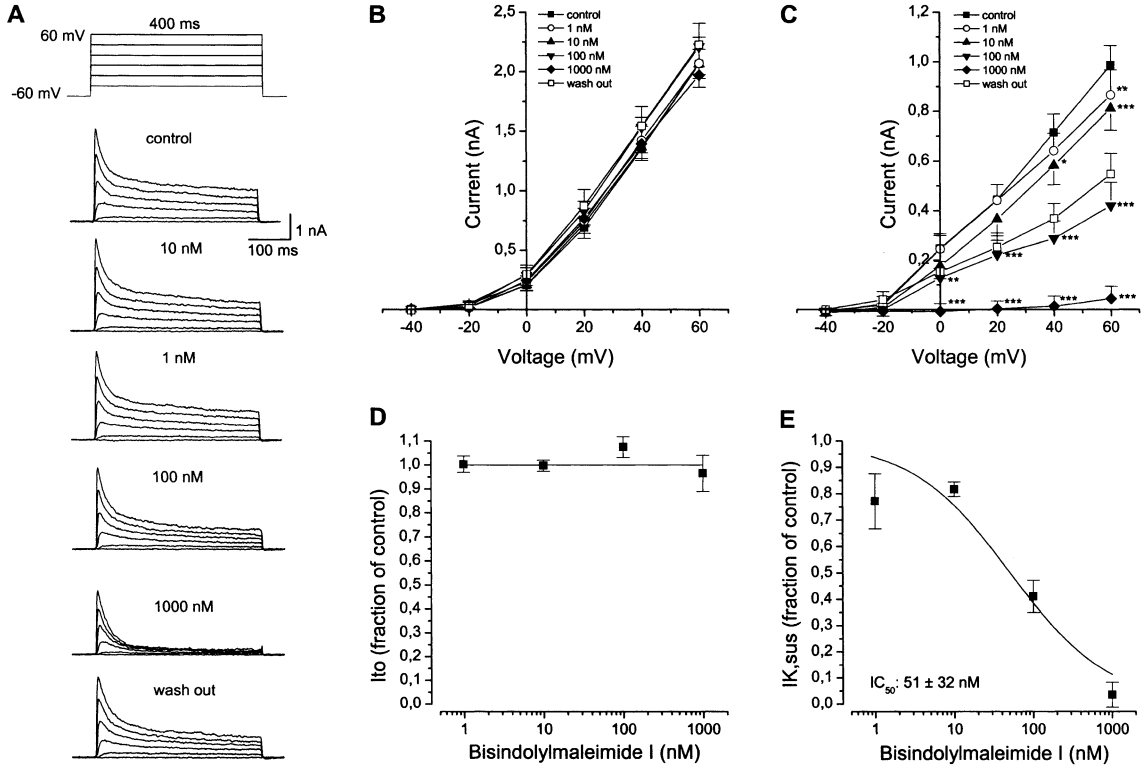

Fig. 3. Effects of bisindolylmaleimide I (BIM I) on outward K^+ currents. (A) Raw K^+ current records from a ventricular myocyte treated with bisindolylmaleimide I (1, 10, 100 and 1000 nM). Currents were elicited from the holding potential of −60 mV to test potential (from −40 to 60 mV) in 20 mV increments, as indicated in the top panel. (B) Current–voltage relationship in the absence and presence of bisindolylmaleimide I for I_{to} and (C) $I_{K,sus}$ (two-way RM ANOVA, $*P<0.05$, $**P<0.01$, $***P<0.001$). (D) Concentration–response curves for effect of bisindolylmaleimide I on I_{to} and (E) $I_{K,sus}$. The amplitudes of the currents at +60 mV were used as the index response. The curves were fitted with the Hill equation (see Materials and methods).

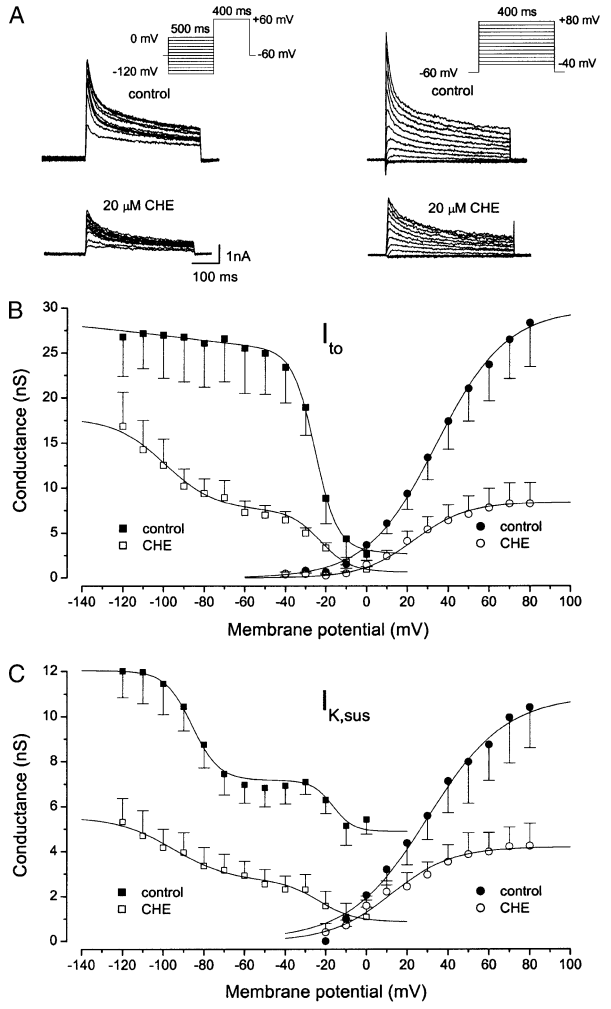

Fig. 4. Effects of chelerythrine (CHE) on the voltage dependence of the steady-state inactivation and activation. (A) Representative current traces from inactivation (left) and activation (right) experiments before (upper traces) and after (lower traces) application of 20 μM chelerythrine. The protocol used is shown in the inset. (B) Steady-state inactivation (left) and activation (right) curves for I_{to} and (C) $I_{K,sus}$ before and after application of chelerythrine. The curves through each data point represent the best fit by the sum of two Boltzmann equations (see Materials and methods).

ing the slope factor (k) and half-maximal inactivation potential ($V_{0.5}$) (Table 1). Chelerythrine (20 μM) had also statistically significant, but small, effect on the slope factor of second I_{to} component ($I_{to,b}$).

As shown in Fig. 4C, $I_{K,sus}$ is also, as suggested previously by Himmel et al. (1999), composed of more than one component: by one that is inactivated at relatively negative potentials ($I_{K,sus,a}$) and another that is inactivated by voltage up to 0 mV ($I_{K,sus,b}$) and by a component that is resistant to inactivation. The effects of chelerythrine treatment on the slope factors of a and b components were statistically significant (Table 1), whereas it had no influence on $V_{0.5}$ of these components. Chelerythrine treatment notably decreased the conductance of $I_{K,sus}$ over the whole range of inactivation experiments (Fig. 4C).

Table 1
Effects of chelerythrine (CHE) and bisindolylmaleimide I (BIM I) on parameters of steady-state inactivation curve of I_{to} and $I_{K,sus}$

		Parameter	Control	Treatment
CHE	I_{to}	k_a	− 51.45 ± 11.58	− 12.35 ± 0.82[a]
		$V_{0.5,a}$ (mV)	− 91.61 ± 0.60	− 99.20 ± 1.45[a]
		k_b	− 4.00 ± 0.41	− 7.37 ± 1.25[a]
		$V_{0.5,b}$ (mV)	− 21.67 ± 3.97	− 22.76 ± 3.74
	$I_{K,sus}$	k_a	− 6.57 ± 1.03	13.70 ± 1.09[b]
		$V_{0.5,a}$ (mV)	− 85.88 ± 4.28	− 95.19 ± 5.09
		k_b	− 5.00 ± 0.54	− 8.80 ± 1.33[a]
		$V_{0.5,b}$ (mV)	− 16.54 ± 3.73	− 23.21 ± 2.01
BIM I	I_{to}	k_a	− 29.92 ± 10.55	− 19.39 ± 9.67
		$V_{0.5,a}$ (mV)	− 80.60 ± 8.80	− 86.90 ± 4.23
		k_b	− 3.85 ± 0.38	− 4.68 ± 0.23[a]
		$V_{0.5,b}$ (mV)	− 22.01 ± 0.73	− 20.46 ± 1.28
	$I_{K,sus}$	k_a	− 7.15 ± 2.06	− 11.91 ± 1.03
		$V_{0.5,a}$ (mV)	− 84.45 ± 2.34	− 91.81 ± 1.03
		k_b	− 8.50 ± 1.26	− 8.39 ± 1.41
		$V_{0.5,b}$ (mV)	− 20.75 ± 2.52	− 21.60 ± 5.29

Data are expressed as mean ± S.E.M. ($n = 5$ cells). $V_{0.5,a}$ and $V_{0.5,b}$, the potential at which the conductance is half-maximally inactivated; k_a and k_b, the slope factor of the respective current components. Statistical significance: [a]$P < 0.05$, [b]$P < 0.01$ (paired t-test) as compared with the respective control.

Representative current traces elicited by activation protocol before and after application of 20 μM chelerythrine are shown in Fig. 4A (right traces). Pooled data are plotted in Fig. 4B (I_{to}, right curve) and Fig. 4C ($I_{K,sus}$, right curve)($n = 5$). Application of chelerythrine shifts conductance curves of I_{to} and $I_{K,sus}$ slightly (11.0 and 16.8 mV, respectively) to the left (Table 2), but had no influence on slope factors (k). Chelerythrine significantly inhibited the maximum conductance of I_{to} from 28.4 ± 10.8 to 11.2 ± 3.8 nS ($P < 0.01$) and $I_{K,sus}$ from 10.5 ± 4.3 to 4.3 ± 2.2 nS ($P < 0.01$).

An example of the effects of 100 nM bisindolylmaleimide I on the outward K^+ currents elicited by the inactivation protocol is shown in Fig. 5A (left traces). Application of bisindolylmaleimide I slightly increased the conductance of I_{to}, and the effect on the shape of the inactivation curve was minor ($n = 5$). The effect of bisindolylmaleimide I on $I_{K,sus}$ inactivation curve parallels that of chelerythrine,

Table 2
I_{to} and $I_{K,sus}$ steady-state activation parameters before and after chelerythrine (CHE) or bisindolylmaleimide I (BIM I) application

		Parameter	Control	CHE
CHE	I_{to}	k	17.02 ± 0.66	12.86 ± 0.95
		$V_{0.5}$ (mV)	34.26 ± 3.90	23.26 ± 1.31[a]
	$I_{K,sus}$	k	17.98 ± 0.61	14.90 ± 1.93
		$V_{0.5}$ (mV)	28.20 ± 2.01	11.40 ± 5.07[b]
BIM I	I_{to}	k	16.94 ± 0.77	17.44 ± 0.60
		$V_{0.5}$ (mV)	25.48 ± 2.90	27.92 ± 2.91[a]
	$I_{K,sus}$	k	20.30 ± 0.59	16.60 ± 1.77
		$V_{0.5}$ (mV)	33.18 ± 1.8	17.26 ± 4.2[a]

Data are expressed as mean ± S.E.M. ($n = 5$ cells). $V_{0.5}$, the potential at which the conductance is half-maximally activated; k, slope factor. Statistical significance: [a]$P < 0.05$, [b]$P < 0.01$ (paired t-test) as compared with the respective control.

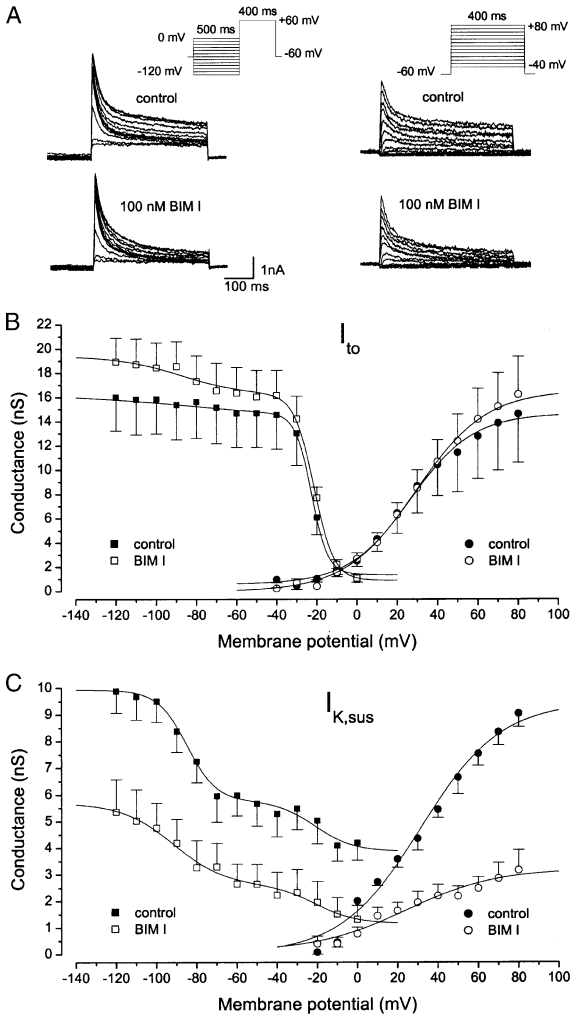

Fig. 5. Effects of bisindolylmaleimide I (BIM I) on the voltage dependence of the steady-state inactivation and activation. (A) Representative current traces from inactivation (left) and activation (right) experiments before (upper traces) and after (lower traces) addition of 100 nM bisindolylmaleimide I. The protocol used is shown in the inset. (B) Mean steady-state activation (left) and inactivation (right) curves for I_{to} and (C) $I_{K,sus}$ in control and bisindolylmaleimide I treated myocytes. The curves through each data point represent the best fit by the sum of two Boltzmann equations (see Materials and methods).

except that the influence on slope factors (k_a, k_b) were statistically nonsignificant (Table 1).

Representative current traces elicited by the activation protocol before and after application of 100 nM bisindolylmaleimide I are shown in Fig. 5A (right traces). The mean values of $V_{0.5}$ and k before and after application of bisindolylmaleimide I are shown in Table 2. Bisindolylmaleimide I shifted the mid-point of the activation curve of I_{to} by 2.4 mV (Fig. 5B), but although the effect is statistically significant, it can be counted as being negligibly small. The mid-point of the activation curve of $I_{K,sus}$ was shifted by -15.9 mV. Bisindolylmaleimide I had no statistically significant effect on the slope factors. Bisindolylmaleimide I significantly inhibited the maximum conductance of $I_{K,sus}$ (from 9.0 ± 1.2 to 3.3 ± 1.6 nS, $P = 0.001$) but had no statistically significant effect on the conductance of I_{to} (14.6 ± 9.0 nS in control and 16.2 ± 7.0 nS after bisindolylmaleimide I application, $P > 0.05$).

3.4. Effect of chelerythrine and bisindolylmaleimide on I_{K1}

When the myocytes were exposed to different concentrations of the protein kinase C inhibitor, the amplitude of I_{K1} was reduced ($n = 5$, data not shown). In the presence 20 μM chelerythrine and 100 nM bisindolylmaleimide I, mean current amplitude at -130 mV was decreased statistically significantly from -2.5 ± 0.4 to -2.1 ± 0.3 nA and from -2.3 ± 0.2 to 2.0 ± 0.2 nA, respectively. However, at -70 mV, the effect on current amplitude was minor and statistically nonsignificant. The effects were not reversed during a 4-min washout.

3.5. Effect of chelerythrine and bisindolylmaleimide on I_{Ca}

Chelerythrine reduced I_{Ca} slightly but only with the highest concentration (50 μM) (at 0 mV from -1.5 ± 0.1 to -1.2 ± 0.1 nA, $n = 5$, data not shown). This response recovered completely by a 4-min washout. Bisindolylmaleimide I had little or no effect on I_{Ca} in the concentration range we used ($n = 5$, data not shown).

3.6. Effects of chelerythrine and bisindolylmaleimide I on I_{to} and $I_{K,sus}$ in ruptured patch-clamp configuration

To solve whether the effects of chelerythrine and bisindolylmaleimide I on K^+ are mediated channels by inhibition of basal protein kinase C activity, or it is caused by direct ion channel actions of these substances, we studied the effect of protein kinase C inhibitors on I_{to} and $I_{K,sus}$ also in ruptured patch-clamp configuration at 22 °C. In these conditions, basal protein kinase C activity should be much lower than with perforated patch-clamp at 29 °C. As shown in Fig. 6A,B, application of 20 μM chelerythrine inhibited I_{to} ($63 \pm 6\%$ vs. $31 \pm 8\%$ in perforated experiments at $+60$ mV) and $I_{K,sus}$ ($28 \pm 6\%$ vs. $59 \pm 6\%$ in perforated experiments at $+60$ mV) significantly also in these conditions ($n = 5$). $I_{K,sus}$ recovered partially during a 4-min washout, whereas I_{to} inhibition did not, as with perforated patch-clamp experiments. Also, bisindolylmaleimide I inhibited $I_{K,sus}$ (Fig. 6C) ($n = 5$) but to a lesser degree than in perforated patch-clamp configuration ($28 \pm 6\%$ vs. $59 \pm 6\%$ at $+60$ mV, respectively).

3.7. Effects of bisindolylmaleimide V, calphostin C, 12-O-tetradecanoyl phorbol 13-acetate and 1-oleoyl-2-acetyl-sn-glycerol on outward K^+ currents

We also studied the effects of bisindolylmaleimide V, calphostin C, TPA and OAG on I_{to} and $I_{K,sus}$ in perforated patch-clamp configuration. Bisindolylmaleimide V, which is

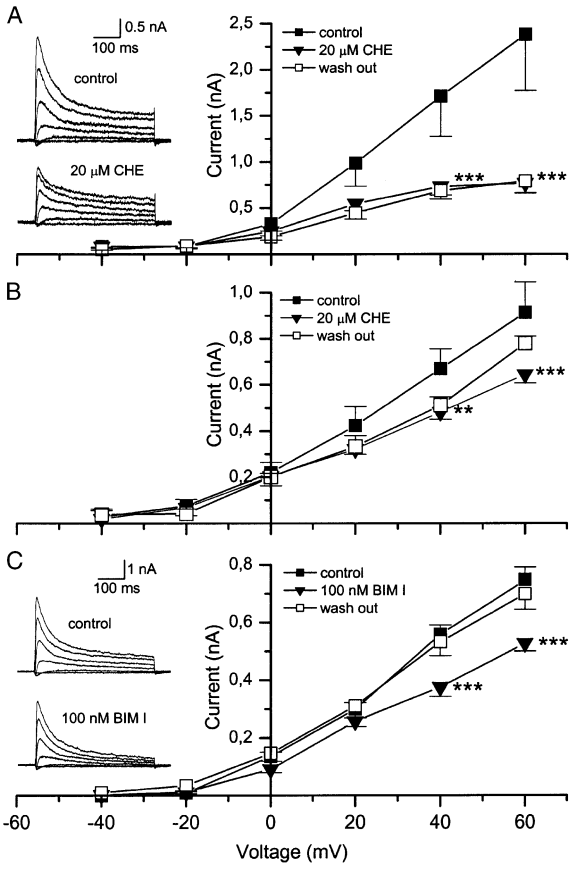

Fig. 6. Effects of chelerythrine (CHE) and bisindolylmaleimide I (BIM I) on outward K^+ currents in ruptured patch-clamp configuration at 22 °C. The myocytes were held at −60 mV and depolarized from −40 to +60 mV in 20 mV increments. Current–voltage relationship of (A) I_{to} and (B) $I_{K,sus}$ in the absence and presence of 20 μM chelerythrine (two-way RM ANOVA, $^{**}P<0.01$, $^{***}P<0.001$). (C) Current–voltage relationship of $I_{K,sus}$ in the absence and presence of 100 nM bisindolylmaleimide I. Insets show examples of current tracings before and after superfusion with drug.

an inactive analog of bisindolylmaleimide I, is useful as a negative control compound for protein kinase C inhibition by bisindolylmaleimide I. As bisindolylmaleimide I, also bisindolylmaleimide V inhibited $I_{K,sus}$ significantly in a concentration-dependent manner (Fig. 7B) but had no effect on I_{to} (Fig. 7A) ($n=5$). The IC_{50} value for inhibition of $I_{K,sus}$ by bisindolylmaleimide V (399 ± 82 nM) (Fig. 7C) is rather high compared to IC_{50} value of bisindolylmaleimide I (51 ± 32 nM). This difference may be due to difference in the affinity between drug and channel.

Calphostin C is known to inhibit protein kinase C potently and highly selectively by binding to the regulatory domain (Kobayashi et al., 1989). In these experiments, the microscope light was turned on, since calphostin C inhibits protein kinase C only in the presence of light (Bruns et al., 1991). Application of calphostin C had no effect on outward K^+ currents ($n=5$, data not shown).

OAG and TPA, which are known to activate protein kinase C via a direct mechanism, did not increase the outward K^+ currents ($n=2–3$, data not shown) but rather decreased them especially with high concentrations (100 nM TPA and 100 μM OAG). These effects recovered partially during a 4-min washout.

3.8. Use-dependency of chelerythrine and bisindolylmaleimide actions on outward K^+ currents

As shown in Fig. 8A, the amplitude of I_{to} stayed unchanged under control conditions. In the presence of 20 μM chelerythrine ($n=3$), the amplitude of I_{to} decreased progressively and significantly ($P<0.01$ starting from pulse number 2 compared to control). However, at first pulse, the I_{to} amplitude was decreased only a little (2.2 ± 0.3 nA vs. 2.8 ± 0.3 nA in control). These results indicate that cheler-

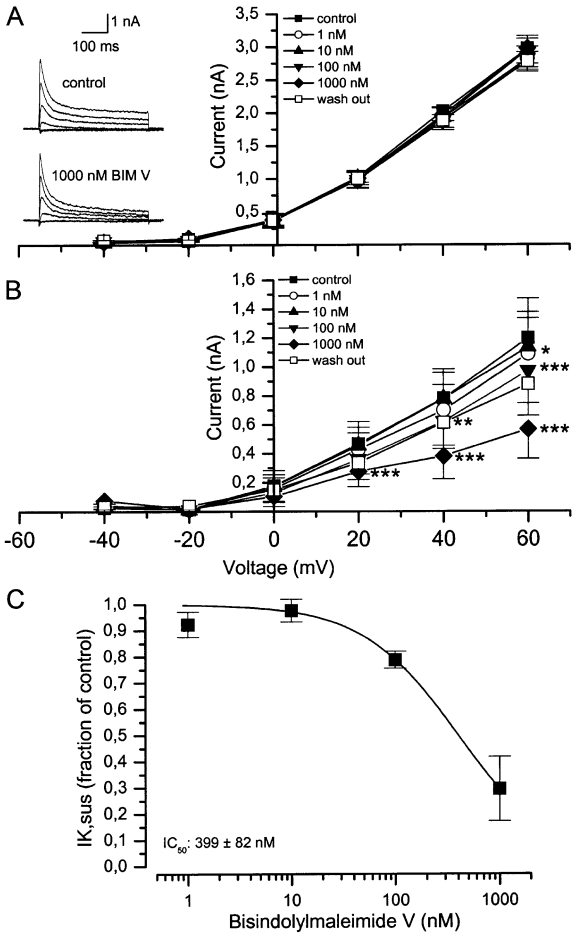

Fig. 7. Effects of bisindolylmaleimide V (BIM V) on outward K^+ currents. Currents were elicited by test pulses stepping from −40 to +60 mV in 20 mV increments from the holding potential of −60 mV. Current–voltage relationship in the absence and presence of bisindolylmaleimide V (1, 10, 100 and 1000 nM) for (A) I_{to} and (B) $I_{K,sus}$ (two-way RM ANOVA, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$). Examples of original current tracings before and after superfusion with 1000 nM bisindolylmaleimide V are shown in the inset. (C) Concentration–response curves for the inhibitory effect of bisindolylmaleimide V on $I_{K,sus}$. The amplitudes of $I_{K,sus}$ at depolarizing step to +60 mV were used to construct the concentration–response curve, and the amplitudes are shown as the fraction of the control. The curve was fitted with the Hill equation (see Materials and methods).

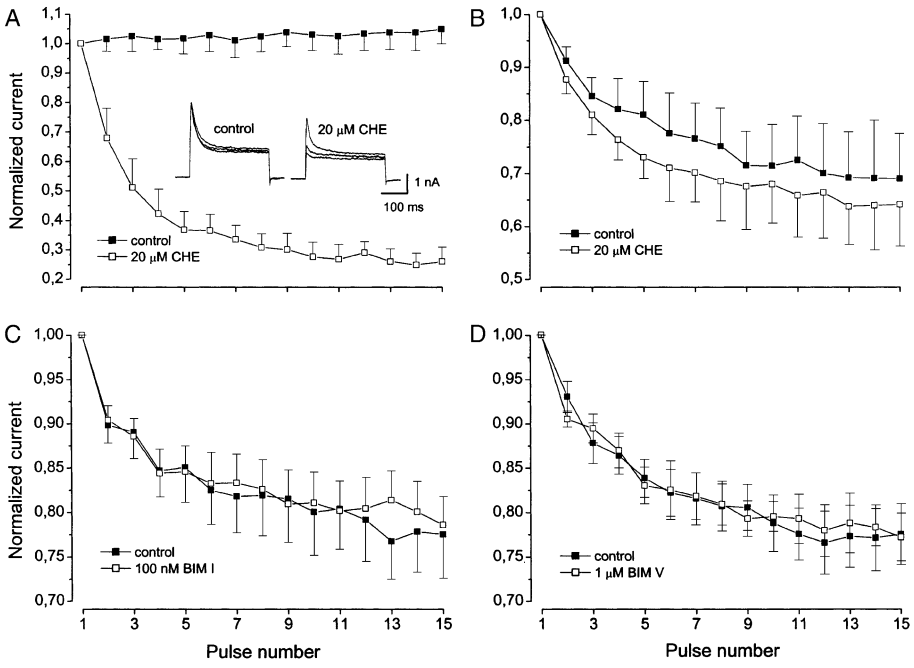

Fig. 8. Use-dependency of (A) I_{to} inhibition by 20 μM chelerythrine (CHE). Inset shows examples of original current records before and after superfusion with chelerythrine. Only currents at first, third and fifteenth pulses are shown. Use-dependency of $I_{K,sus}$ inhibition by (B) 20 μM chelerythrine, (C) 100 nM bisindolylmaleimide I (BIM I) and (D) 1 μM bisindolylmaleimide V (BIM V). Fifteen repetitive pulses from holding potential of −60 to +60 mV for 300 ms were applied at 1 Hz. The amplitude of current at every pulse was normalized by the current amplitude at the first pulse.

ythrine blocks I_{to} preferentially in the open or inactivated state. The effect of chelerythrine on $I_{K,sus}$ was minor, but statistically significantly use-dependent ($P<0.05$ starting from pulse 2 compared to control) (Fig. 8B). Application of bisindolylmaleimide I ($n=5$) and bisindolylmaleimide V ($n=5$) had no effect on use-dependency of $I_{K,sus}$ compared to control (Fig. 8C,D); thus, the block of $I_{K,sus}$ by bisindolylmaleimide I and bisindolylmaleimide V is not dependent on channel openings.

4. Discussion

In the present study, we investigated the influence of protein kinase C inhibitors on the action potential duration and voltage-activated K^+ and Ca^{2+} currents in isolated rat ventricular myocytes. The experiments showed that action potential duration is prolonged in response to application of chelerythrine or bisindolylmaleimide I (Fig. 1A,B), two commonly used protein kinase C inhibitors and that this is due to the modulation of ionic channels, especially voltage-dependent outward K^+ channels.

In this study, application of chelerythrine or bisindolylmaleimide I led to a concentration-dependent decrease in the outward K^+ currents (Figs. 2 and 3). In the presence of chelerythrine, both $I_{K,sus}$ and I_{to} were attenuated. However, the IC_{50} values for inhibition of these currents were clearly distinct (20 ± 6 and 43 ± 9 μM, respectively). Furthermore, the recoveries during washout were different: $I_{K,sus}$ recovered noticeably during a 4-min washout while I_{to} recovered hardly at all (Figs. 4 and 6). Thus, the effects of chelerythrine on $I_{K,sus}$ and I_{to} seemed to be independent of each other. Like chelerythrine, bisindolylmaleimide I also inhibited $I_{K,sus}$ concentration-dependently, but bisindolylmaleimide I treatment had no effect on I_{to}. On the basis of these findings, it is impossible to say whether the effects of chelerythrine and bisindolylmaleimide I on outward K^+ currents are mediated by inhibition of basal protein kinase C activity or by a protein kinase C-independent pathway.

A series of experiments were performed to solve that problem. The following findings support the conclusion of protein kinase C-independent effects. (a) The effects of chelerythrine and bisindolylmaleimide I on I_{to} and $I_{K,sus}$ were almost the same in perforated (Figs. 2 and 3) and ruptured (Fig. 6) patch-clamp configuration. In ruptured configuration, protein kinase C activity is presumably reduced when compared to that in perforated configuration due to dialysis of important cytosolic components. In addition, ruptured patch experiments were performed at lower temperature, which decreases enzyme activity. If the responses were protein kinase C-dependent, the effects of chelerythrine and bisindolylmaleimide I should be dramatically lowered or totally absent in ruptured patch-clamp conditions. However, this was not the case. (b) Bisindolylmaleimide V, which is an inactive analog of bisindolylmaleimide I, inhibited $I_{K,sus}$ dose-dependently, albeit the IC_{50} was higher. This clearly indicates that substances analogous to bisindolylmaleimide V, like bisindolylmaleimide I, have protein kinase C-independent effects on $I_{K,sus}$. (c) Calphostin C, which inhibits protein kinase C in a highly specific

manner by binding to regulatory domain of protein kinase C, failed to produce any effect on outward K^+ currents. Thus, basal protein kinase C activity does not seem to modulate these currents. (d) Direct protein kinase C activators TPA and OAG did not stimulate outward K^+ currents but tended to decreased them. Similar findings have been reported previously in the literature (Kobayashi et al., 1989; Nakamura et al., 1997; Shimoni, 1999). It is not probable that both activation and inhibition of protein kinase C system would produce the same response. Based on the material shown in the present paper, K^+ current inhibition caused by chelerythrine and bisindolylmaleimide I is independent of protein kinase C activity.

Also, by comparing the IC_{50} values for inhibition of I_{to} and $I_{K,sus}$ (43 and 20 μM, respectively) by chelerythrine obtained in this study with IC_{50} value (0.66 μM) reported for the inhibition of purified rat protein kinase C (Herbert et al., 1990), it may be assumed that the observed effects on K^+ currents are independent of protein kinase C inhibition, since IC_{50} values differ greatly. On the other hand, if we compare the IC_{50} value for inhibition of $I_{K,sus}$ by bisindolylmaleimide I (51 nM) with the IC_{50} value for protein kinase C inhibition by bisindolylmaleimide I (10 nM, purified bovine protein kinase C) (Toullec et al., 1991), we can observe that the bisindolylmaleimide I concentration that modulates $I_{K,sus}$ in rat ventricular myocytes is rather similar to that required to inhibit the protein kinase C activity. In both cases, the concentrations that inhibit outward K^+ currents in this study are comparable to those commonly used in experiments investigating the role of protein kinase C.

Use-dependency experiments (Fig. 8) showed that chelerythrine had only a small effect on I_{to} at rest, and also that it inhibits I_{to} in a use-dependent manner. These findings indicate that decrease in I_{to} is not caused by protein kinase C inhibition, but chelerythrine interacts directly with the open or inactivated state of ion channels carrying I_{to}. In addition, chelerythrine does not seem to dissociate from channel once attached and thus the effect is accumulating. Also, $I_{K,sus}$ was inhibited use-dependently following chelerythrine application, but the effect was small, albeit statistically significant. It is possible that this effect is caused by the effect of chelerythrine on I_{to}: the pulse duration used in these experiments is not long enough to totally inactivate I_{to}, and thus, changes in the residual I_{to} current at the end of the pulse could lead to a small error. We can conclude that both bisindolylmaleimide I and chelerythrine affect $I_{K,sus}$ by a mechanism other than open channel block. One possible mechanism is closed channel block, and in addition, it is possible that the response is due to an inhibition of a kinase other than the protein kinase C.

We also studied the voltage dependence of I_{to} and $I_{K,sus}$ inactivation and activation following chelerythrine and bisindolylmaleimide I treatment. Application of chelerythrine had an exceptional influence on the shape of the inactivation curve of I_{to} (Fig. 4). At first glance, it seems likely that there are at least two possible inactivation states for I_{to} channels, and during chelerythrine treatment, a part of the channels changes into a state, which inactivates at voltages below -60 mV (Fig. 4B). Another likely explanation is that since there are at least two K^+ channel proteins with I_{to}-like properties in rat ventricular myocytes (Nerbonne, 2000), chelerythrine treatment changes the inactivation properties of definite channel subtype. These interpretations could also explain the observed decrease of I_{to} conductance (Fig. 4B) measured from the holding potential of -60 mV. Part of the I_{to} channels are inactivated at -60 mV (holding potential), and thus the decrease of I_{to} conductance could be followed from increased channel inactivation and from the thereby decreased channel availability. However, these interpretations seem to be incorrect. The most likely explanation for this phenomena is that the decrease in the conductance of I_{to} at voltages below -60 mV is caused by an accumulating channel block (Figs. 2C and 6A), which is affecting open or inactivated channels (Fig. 8A). More and more channels were blocked as the experiment was carried out, and finally, at voltages below -60 mV, only unblocked channels inactivated. This kind of block also explains the decrease of I_{to} conductance in activation experiments. As expected, bisindolylmaleimide I application had no significant effect on the voltage dependence of I_{to} inactivation or activation (Fig. 5B). Both chelerythrine and bisindolylmaleimide I affected the level of $I_{K,sus}$ inactivation curve, but had only a minor effect on the Boltzmann parameters (Table 1). On the basis of these findings, we suggest that chelerythrine and bisindolylmaleimide I do not change the voltage dependence of I_{to} and $I_{K,sus}$ activation and inactivation but modulate single-channel open probability or single-channel conductance, and thus attenuate whole-cell conductance.

Chelerythrine or bisindolylmaleimide I attenuated also the hyperpolarization-activated I_{K1}, but only at potentials much below the normal resting membrane potential. Thus, we conclude that changes in I_{K1} did not contribute to the observed prolongation of the action potential duration. We also studied the effects of chelerythrine and bisindolylmaleimide I on I_{Ca} and found that they had no marked influence. This finding is in concert with the finding of Thomas et al. (1997). The observed inhibitory effect at high (50 μM) concentration of chelerythrine may be attributed to inhibition of kinases other than the protein kinase C. For example, Herbert et al. (1990) reported that chelerythrine inhibits protein kinase A, which is involved in the regulation of cardiac Ca^{2+} channels (Trautwein and Heschler, 1990), with the IC_{50} value of 170 μM.

In conclusion, our data clearly show that the protein kinase C inhibitors chelerythrine and bisindolylmaleimide I caused a prolongation of the action potential duration in single adult rat ventricular myocytes mainly by reducing the outward K^+ currents. However, the reduction is not caused by basal protein kinase C inhibition, but is due to "nonspecific" effects of these drugs. The nonspecific effects of

these drugs should be taken into account when studying and interpreting protein kinase C actions on the heart, or more generally on ion channels, and when using these protein kinase C inhibitors.

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