Time-dependent block of the slowly activating delayed rectifier K$^+$ current by chromanol 293B in guinea-pig ventricular cells

[1]Susumu Fujisawa, [1]Kyoichi Ono & *,[1]Toshihiko Iijima

[1]Department of Pharmacology, Akita University School of Medicine, Hondoh 1-1-1, Akita 010-8543, Japan

1   The slowly activating delayed rectifier K$^+$ current (I_{Ks}) was recorded in single myocytes dissociated from guinea-pig ventricles and the mechanism underlying the block of I_{Ks} by a chromanol derivative, 293B, was investigated.

2   In the presence of 1–100 μM 293B, activation phase of I_{Ks} was followed by a slower decay during 10 s depolarizing pulses. Both the rate and extent of the decay were increased in a concentration-dependent manner.

3   The relationship between the concentration of 293B and the block showed a Hill's coefficient of approximately 1. The half-inhibitory concentration was approximately 3.0 μM and did not differ significantly at various membrane potentials from +20 to +80 mV.

4   A mathematical model for the 293B block was constructed on the basis of multiple closed and open states for the I_{Ks} channels, and the blocking rate was calculated by fitting the model to the original current traces. The blocking rate constant showed a linear function with the 293B concentration, indicating 1 : 1 binding stoichiometry. At +80 mV the blocking rate was 4×10^4 M^{-1} s^{-1} and the unblocking rate was 0.2 s^{-1}.

5   The results indicate that 293B is an open channel blocker with relatively smaller blocking rate than those reported so far for time-dependent blockade of various ionic channels.
British Journal of Pharmacology (2000) **129**, 1007–1013

Keywords: Chromanol derivative; 293B; delayed rectifier K$^+$ current; slowly activating I_K; KvLQT1; IsK regulator; minK

Abbreviations: I_K, delayed rectifier K$^+$ current; I_{Kr} rapidly activating delayed rectifier K$^+$ current; I_{Ks}, slowly activating delayed rectifier K$^+$ current

Introduction

The delayed rectifier K$^+$ current (I_K) in cardiac cells consists of fast and slow components, i.e., the rapidly activating I_K (I_{Kr}) and the slowly activating I_K (I_{Ks}) (Sanguinetti & Jurkiewicz, 1990). The currents play a role for repolarizing the membrane after the plateau of the action potential. Since the prolongation of the plateau duration is thought to cause the prolongation of refractoriness, a number of pharmacological agents, which block I_K, has been developed to achieve antiarrhythmic effect. For example, class III antiarrhythmic agents such as d-sotalol, E4031 and dofetilide are thought to block I_{Kr}, thereby causing prolongation of the action potential duration (Roden, 1993; Spector et al., 1996). However, class III antiarrhythmic agents targeted to I_{Kr} often cause an excessive prolongation, thereby leading to torsades de pointes arrhythmias (Roden, 1993).

On the other hand, it has been shown that a chromanol derivative, 293B, blocks I_{Ks} without affecting I_{Kr} or L-type Ca^{2+} current (Busch et al., 1996; Bosch et al., 1998). Since I_{Kr} and I_{Ks} possess different gating properties and voltage dependency (Sanguinetti & Jurkiewicz, 1990), it may be possible that the I_{Ks} block elicits antiarrhythmic action without unfavourable property observed in I_{Kr} blockers (Roden, 1993; Spector et al., 1996). More recently, Loussouarn et al. (1997) demonstrated that 293B is a potent blocker of human KvLQT1 channels and binds to the channel protein itself but not to the IsK (also termed minK) regulator. They showed that 293B blocks KvLQT1 in time- and voltage-dependent manner, and that the rate of the block differs between KvLQT1 current and the current expressed by KvLQT1 plus IsK regulator. The finding indicates that the blocking rate or voltage-dependency

might be different in native I_{Ks}, which consists of KvLQT1 and IsK regulator with unknown stoichiometry (Barhanin et al., 1996; Sanguinetti, et al., 1996). However, systematic analysis for 293B block of native I_{Ks} such as voltage-dependency and the block kinetics has not been carried out. In the present study, therefore, we investigated the effects of 293B on I_{Ks} in guinea-pig ventricular cells, and the time-dependency of the blocking rate was determined for 293B.

Methods

Cell isolation

Ventricular cells were isolated from hearts of guinea-pig (300–400 g body weights) using the enzymatic dissociation technique (Isenberg & Klöckner, 1982; Shinbo & Iijima, 1997). Guinea-pigs were anaesthetized with pentobarbital (50 mg kg^{-1}) and the chest was opened under the artificial respiration. The heart was dissected out and the ascending aorta was cannulated to start coronary perfusion with the control Tyrode solution. After perfusing with 100 ml Ca^{2+}-free Tyrode solution, the perfusate was switched to a Ca^{2+}-free Tyrode solution containing collagenase (0.02%, Wako, Osaka, Japan), and the heart was digested for about 30 min. Then the heart was rinsed with a high K$^+$, low Cl$^-$ storage solution. The left ventricle was dissected from the digested heart, and stored in the storage solution at 4°C for later use.

A small piece of the ventricular tissue was dissected and gently agitated in the recording chamber (0.5 ml in volume) filled with the normal Tyrode solution. After the cells had settled on the floor of the recording chamber, they were

*Author for correspondence; E-mail: tiijima@med.akita-u.ac.jp

perfused with normal Tyrode solution at 2–3 ml min^{-1}. Experiments were performed at 36–37°C on rod-shaped quiescent single cells that had clear sarcomere striations.

Solutions and drugs

The control Tyrode solution contained (in mM): NaCl 136.9, KCl 5.4, CaCl$_2$ 1.8, MgCl$_2$ 0.53, NaH$_2$PO$_4$ 0.33, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) 5.0, and glucose 5.5, (pH = 7.4, adjusted with NaOH). The standard external solution was made by adding 0.3 μM nisoldipine to the control Tyrode solution in order to block the L-type Ca^{2+} current. The Ca^{2+}-free external solution was made by omitting CaCl$_2$ from the standard external solution. The high K$^+$, low Cl$^-$ storage solution contained (in mM): taurine 10, oxalic acid 10, L-glutamic acid 70, KCl 25, KH$_2$PO$_4$ 10, EGTA 0.5, glucose 11, and HEPES 10 (pH = 7.4, adjusted with KOH). The pipette solution contained (in mM): K-aspartate 110, KCl 20, K$_2$ATP, K$_2$-creatine phosphate 6, MgCl$_2$ 2, HEPES 5, EGTA 10 (pH = 7.0, adjusted with KOH).

293B was kindly donated by Hoechst Co. Ltd (Frankfurt, Germany). 293B was dissolved in dimethyl sulphoxide (DMSO) as a 10 mM stock solution, and diluted in the bath solution to give each concentration described in the text. The final concentration of DMSO to which cells were exposed was less than 0.03%. E4031, a selective blocker for I$_{Kr}$, was donated by Eisai Co. Ltd (Tokyo, Japan), and dissolved in distilled water as a 1 mM stock solution and was then added to the bath solution to give the concentration as described in the text.

Electrophysiological experiments

The patch clamp technique (Hamill *et al.*, 1981) was used to record whole-cell currents using a patch clamp amplifier (EPC-7, List, Darmstadt, Germany). Patch pipettes were pulled with a micropipette puller (Model P-97, Sutter Instrument Co., Novato, CA, U.S.A.) from glass capillaries (Corning #7052; Warner Instrument Co., CT, U.S.A.), and the electrode resistance ranged 3–5 MΩ. The data were digitized at 2–10 kHz into an IBM-compatible computer (Proside, Tokyo, Japan) using the pClamp software (Axon Instruments, Foster City, CA, U.S.A.).

Statistical values are given in mean ± s.e.mean. Comparisons between two groups were performed by paired or unpaired Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

Results

293B blocks not only I$_{Ks}$ but also I$_{Kr}$

In guinea-pig ventricular cells, not only I$_{Ks}$ but also I$_{Kr}$ exists (Sanguinetti & Jurkiewicz, 1990). In the experiment shown in Figure 1A, the membrane was depolarized to +60 mV from a holding potential of −40 mV in the standard external solution (a) or in the external solution containing 5 μM E4031 (b), and current recordings with different pulse durations were superimposed. In the control, tail current developed more rapidly than time-dependent outward current during the depolarizing pulse, probably due to the presence of I$_{Kr}$. In the presence of E4031, however, the envelope time course of the tail current corresponded closely to the time course of outward current during the depolarizing pulse. This is clearly shown in Figure 1Ac where the ratio of the tail amplitude relative to the
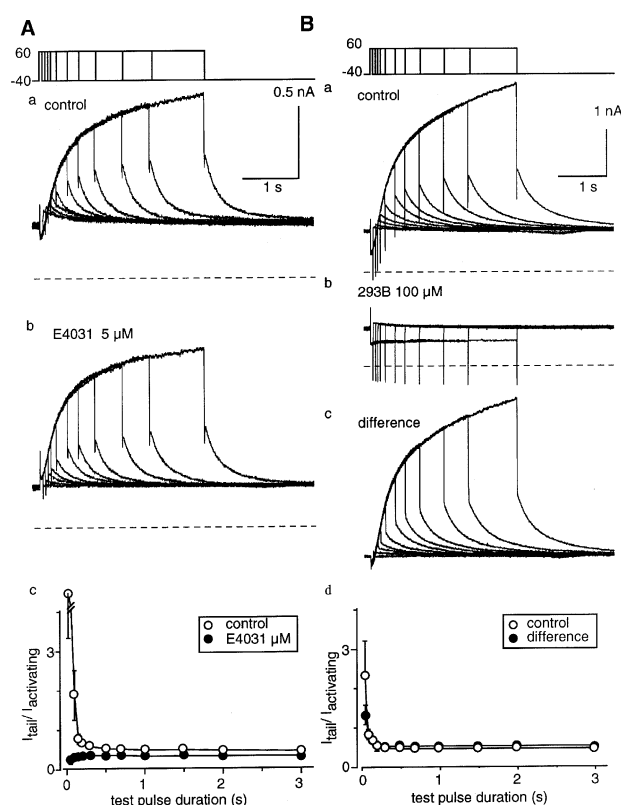

Figure 1 Inhibition of I$_{Ks}$ and I$_{Kr}$ by 293B in guinea-pig ventricular cells. (A) An envelope test of tail currents. Superimposed current traces were obtained when the membrane was depolarized from −40 mV to +60 mV for various duration in the absence (a) and presence (b) of 5 μM E4031. The dotted lines indicate zero current level. (c), The ratio of tail current to time-dependent current. The data are average of six experiments and bars indicate s.e.mean. No bars are shown when the s.e.mean is smaller than the symbol size. (B) An envelope test of tail currents in the absence (a) and presence (b) of 100 μM 293B. The dotted lines indicate zero current level. (c), the 293B-sensitive current was measured by subtracting the current in the presence of 293B (b) from the control (a). (d), The ratio of tail current to time-dependent current. The data are average of three experiments.

outward current during the conditioning pulse, obtained from six cells, was plotted against the pulse duration. The ratio was initially large in the control (open circles) whereas it was almost constant in the presence of E4031 (filled circles). The finding confirmed the selective blockage of I$_{Kr}$ by E4031 (Sanguinetti & Jurkiewicz, 1990).

The same pulse protocol was applied to examine the effect of 293B on I$_{Ks}$ and I$_{Kr}$ (Figure 1B). After obtaining a control envelope test in the standard external solution (Figure 1Ba), 100 μM 293B was applied. In the presence of 293B (Figure 1Bb), both outwardly activating current and tail current were markedly suppressed, except for relatively short pulse durations where small tail currents were still observed. This indicates that the 293B-sensitive current (c), obtained by subtracting the current in the presence of 293B (b) from the control current (a), includes the current component attributable to I$_{Kr}$. In fact, the ratio of the tail amplitude to the outward current (d), measured for the 293B-sensitive current, was not constant during the conditioning pulse and showed an initial peak (filled circles) although in a smaller extent than that of the control (open circles). Thus, 293B appeared to inhibit not only I$_{Ks}$ but also I$_{Kr}$. In order to examine the mechanism of I$_{Ks}$ block by 293B, following experiments were carried out in the presence of 5 μM E4031.
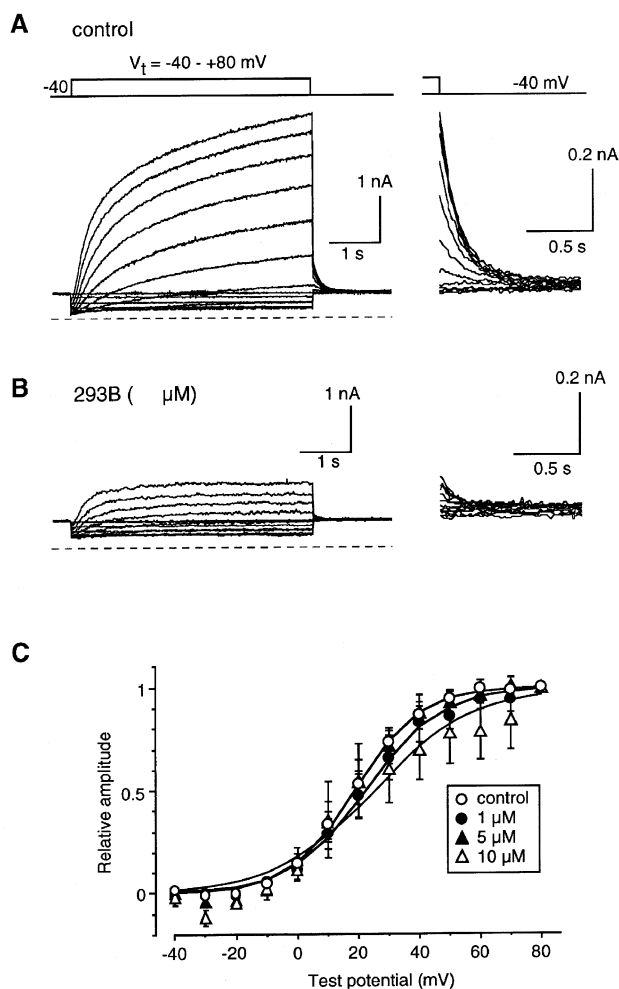
Figure 2 Voltage-dependent activation of I$_{Ks}$. (A) A 5-s depolarizing pulse was applied every 30 s from -40 mV to various test potentials in 10 mV increment. Superimposed current traces were recorded in the standard external solution containing 0.3 μM nisoldipine. Five μM E4031 was added to the external solution to block I$_{Kr}$. The dotted line indicates the zero current level. Right-hand panel; tail currents are shown on an expanded time scale. (B) The same pulse protocol was repeated in the presence of 10 μM 293B. (C) The relationship between the test potential and the tail amplitude at different [293B]$_o$. The amplitude of the tail current was normalized to the maximum amplitude for the given concentration of 293B. Data are average of 4–6 experiments and bars indicate s.e.mean. The smooth curves were drawn by the least squares fit with the Boltzmann equation, I = 1/ (1+exp((V − V$_{1/2}$)/S)), where V$_{1/2}$ and S indicate the half-activation voltage and the slope factor, respectively. For details, see text.

Steady-state activation of I$_{Ks}$

The effects of 293B on the steady-state activation of I$_{Ks}$ were examined. In the experiment shown in Figure 2, a 5 s depolarizing pulse was applied from -40 mV to various potentials in 10 mV increment in the absence (A) and presence of 10 μM 293B (B). To obtain the steady-state activation, the amplitude of the tail current was measured, normalized in reference to the maximum amplitude for the drug concentration given, and plotted against the test potentials (Figure 2C). For each concentration of 293B, four to six cells were examined. The smooth curves were drawn by the least squares fit with the Boltzmann equation,

$$I = 1/(1 + \exp((V - V_{1/2})/S)), \qquad (1)$$

where V$_{1/2}$ and S indicate the half-activation voltage and the slope factor, respectively. On average, the value of V$_{1/2}$ was
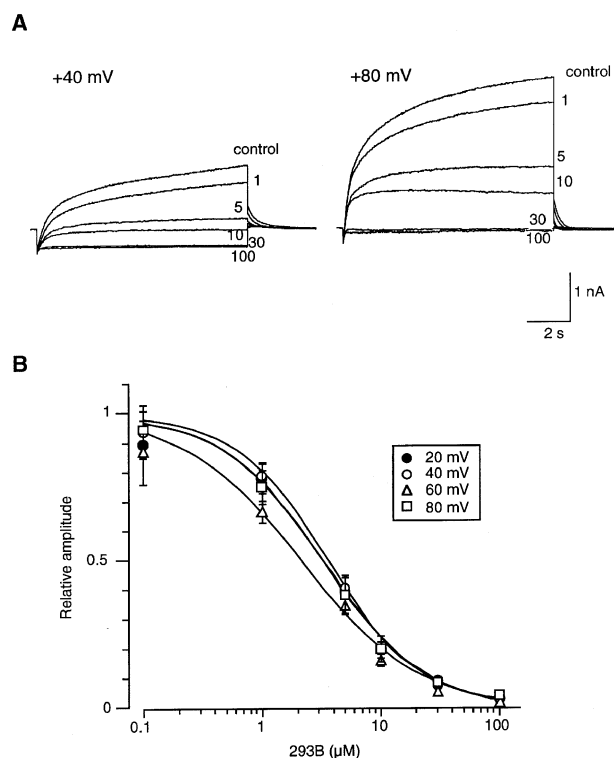

Figure 3 Concentration-dependent inhibition of I$_{Ks}$ by 293B. (A) A 10 s depolarizing pulse from -40 mV to either $+40$ mV (left panel) or $+80$ mV (right panel) was repeated every 1 min. Superimposed current traces were recorded at different [293B]$_o$. Numerals indicate [293B]$_o$ in μM. (B) The relationship between the I$_{Ks}$ amplitude and [293B]$_o$, obtained at different [293B]$_o$. The amplitude of time-dependent outward current during the test pulse was normalized to that of the control current. Data are average of 5–8 experiments, and bars indicate s.e.mean. No bars are shown when the s.e.mean is smaller than the symbol size. Filled circles, $+20$ mV; open circles; $+40$ mV; open triangles, $+60$ mV; open squares, $+80$ mV. The smooth curves were drawn by the least squares fit with the equation, I = 1/(1 + ([293B]$_o$/IC$_{50}$)^n), where IC$_{50}$ and n indicate the half-inhibitory concentration and the Hill's coefficient, respectively. For details, see text.

18.76 ± 3.16 mV ($n=6$) in the control, 23.56 ± 4.92 mV ($n=4$) at 1 μM, 18.70 ± 4.97 mV ($n=5$) at 5 μM, and 27.63 ± 8.30 mV ($n=4$) at 10 μM 293B. The value of S was 10.10 ± 0.78 mV in the control, 10.63 ± 1.90 mV at 1 μM, 10.00 ± 0.67 mV at 5 μM, and 16.03 ± 4.93 mV at 10 μM 293B. Thus, 293B inhibits I$_{Ks}$ without affecting its kinetics.

Concentration-response relationship for the 293B block

Current records obtained at different extracellular concentrations of 293B ([293B]$_o$) were compared at $+40$ mV and $+80$ mV (Figure 3). The blocking effect of 293B became evident around 0.1 μM and was almost saturated at 100 μM. At lower [293B]$_o$ the configuration of I$_{Ks}$ was not obviously altered by 293B. But, increasing [293B]$_o$, a time-dependent inhibition of I$_{Ks}$ was revealed. For example, at 5 or 10 μM 293B the initial activation time course of I$_{Ks}$ were almost superimposable on the control, and thereafter the current was time-dependently depressed.

The [293B]$_o$-block relationship was constructed by measuring the current level near the end of depolarizing pulse. The current amplitudes were normalized referring to those obtained in the absence of 293B, and results were
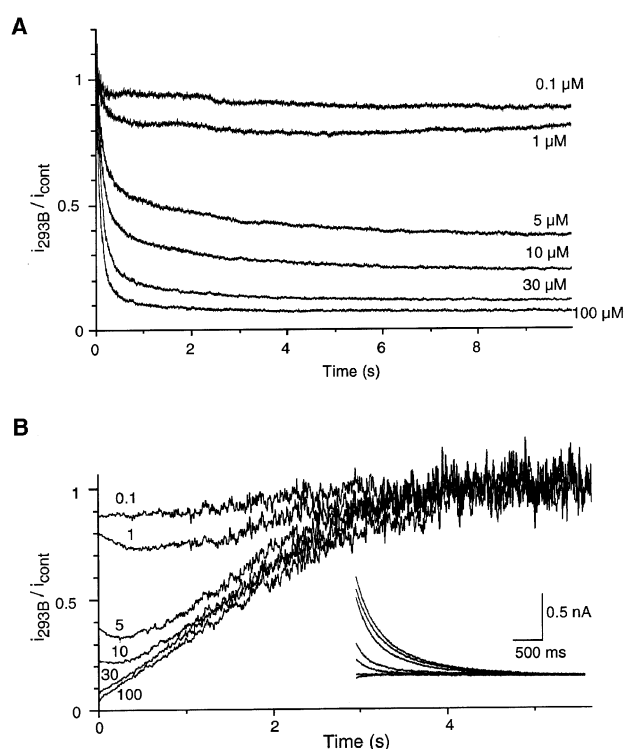
...

A

B

Figure 4 Time-dependent block of I$_{Ks}$ by 293B. (A) A 10 s pulse from −40 mV to +40 mV was applied every 1 min. Time-dependent outward current during a depolarizing step at different [293B]$_o$ was digitally divided by the current in the absence of 293B (i$_{293B}$/i$_{cont}$) and superimposed. (B) The same calculation was performed for tail current. Numerals indicate [293B]$_o$ in μM. In the inset, original traces of tail current are shown.

summarized in Figure 3B. The smooth curves were drawn by the least squares fit with the equation,

$$I = 1/(+([293B]_o/IC_{50})^n), \qquad (2)$$

where IC$_{50}$ and n indicate the half-inhibitory concentration and the Hill's coefficient, respectively. The IC$_{50}$ value was 3.17 ± 0.93 ($n=7$) at +20 mV (filled circles), 3.54 ± 0.57 ($n=5$) at +40 mV (open circles), 2.11 ± 0.39 ($n=8$) at +60 mV (open triangles) and 3.24 ± 0.85 ($n=5$) +80 mV (open squares). The n value was 1.08 ± 0.15 at +20 mV, 1.27 ± 0.16 at +40 mV, 0.91 ± 0.06 at +60 mV and 1.11 ± 0.09 at +80 mV. Thus, no significant voltage-dependency could be detected.

Time-dependent block of I$_{Ks}$ by 293B

To confirm the time-dependent block of 293B, I$_{Ks}$ recorded during a depolarizing step to +40 mV in the presence of 293B was digitally divided by the current recorded in control (i$_{293B}$/i$_{cont}$). This calculation gave a relative amount of unblocked I$_{Ks}$ as a function of time during a depolarizing step (Figure 4A). It was clearly shown that the 293B block became faster and the extent of the block became larger, as [293B]$_o$ was increased.

The same analysis was carried out for the tail current at −40 mV (Figure 4B). In the case of the tail current, i$_{293B}$/i$_{cont}$ should represent the time course of unblock. At 1, 5, 10 μM [293B]$_o$, i$_{293B}$/i$_{cont}$ decreased transiently, and thereafter increased for approximately 4 s. At higher concentrations, i$_{293B}$/i$_{cont}$ showed a continuous increase and became saturated
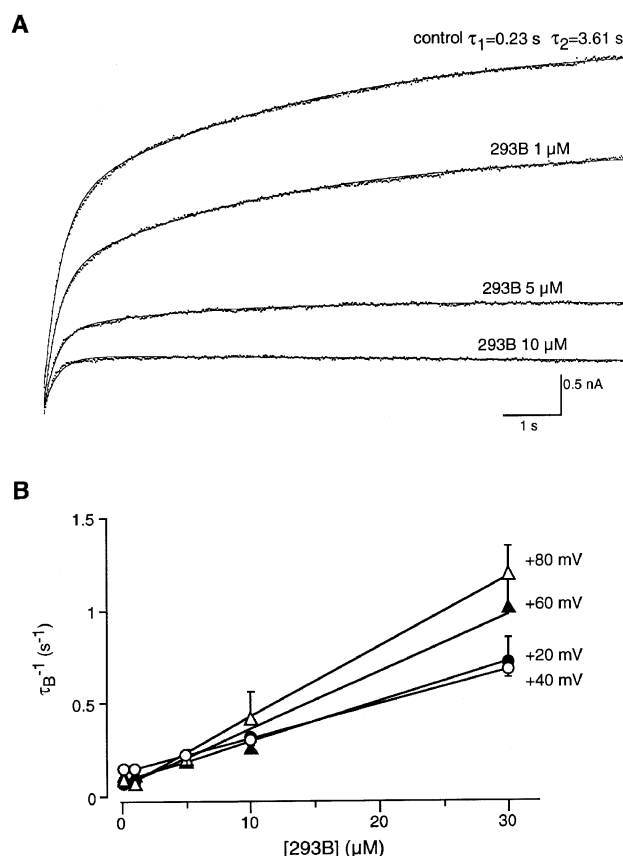
A

B

Figure 5 A mathematical model for the blockade of I$_{Ks}$ by 293B. (A) Superimposed current traces (dotted lines) were obtained by applying a 10 s depolarizing pulse from −40 mV to +60 mV in the absence and presence of 1 or 5 μM 293B. In the control, the current was fitted to the sum of two exponential functions with time constants of 0.23 and 3.61 s (smooth curve). In the presence of 293B, the smooth curves represent the least-squares fit of Equation 6. (See text for details). (B) The relationship between the reciprocal time constant (τ_b^{-1}) and [293B]$_o$. The lines represent the least squares fit at different membrane potentials.

within 4 s. These findings indicate that the time course of recovery was determined by the extent of the block during the preceding depolarization.

Mathematical model for the block of I$_{Ks}$ by 293B

The time-dependent block of I$_{Ks}$ by 293B indicates that 293B acts as an open channel blocker for I$_{Ks}$. In order to determine the block and unblock kinetics, a mathematical model was used. It has been reported that I$_{Ks}$ consists of several closed states and an open state (Balser *et al.*, 1990). Thus, the state transitions between the closed and open states can be described as, C$_1$-C$_2$ - - - - - C$_n$-Open where C(n) is the nth closed conformation. In the present study, the time course of activation of I$_{Ks}$ in the absence of the blocker was well fitted with the sum of two exponential functions, and therefore the time course of the open conformation without 293B can be approximated as

$$z = A[1 - \exp(-t/\tau_1)] + B[1 - \exp(-t/\tau_2)], \qquad (3)$$

where τ_1 and τ_2 are the time constants of the fast and slow components, respectively, and A and B are the proportion of each component in the steady state. Provided that 293B binds to the open state, the blocking model can be described as
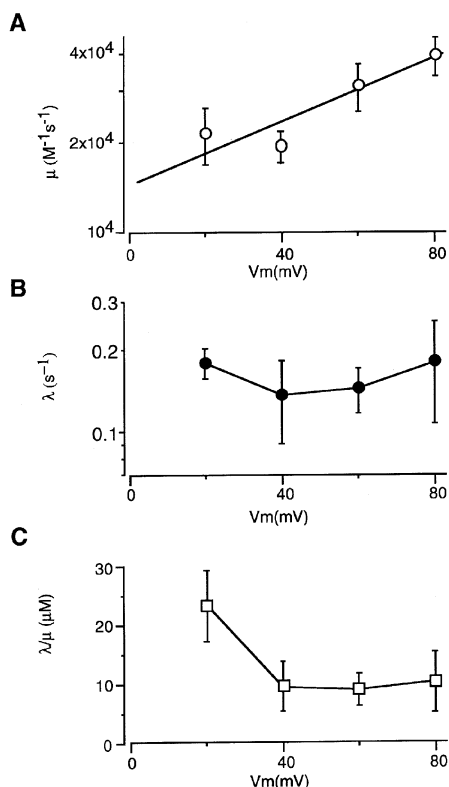
Figure 6 Voltage dependency of the blocking and unblocking rates of 293B. (A) The relationship between the blocking rate (μ) and the membrane potential. The line indicates the least squares fit with a slope of an e-fold increase per 80.9 mV. (B) The relationship between the unblocking rate (λ) and the membrane potential. (C) The relationship between the dissociation constant (λ/μ) and the membrane potential.

$$\overset{\text{x}}{\text{Open}} \underset{\lambda}{\overset{\mu[293B]_o}{\rightleftharpoons}} \overset{\text{y}}{\text{Blocked}}$$

$$dy/dt = \mu[293B]_o x - \lambda y \qquad (4)$$

where x and y represent the open and the block confirmation, respectively. A general equation describing the time-dependent change of the open conformation can be given, assuming that

$$x + y = z \qquad (5)$$

That is, the fraction of channels in the open states (x) and fraction of blocked channels (y) would give the fraction of the channels (z) which are normally open. The solution of these equations for x is given as:

$$x = (A - P) * [1 - \exp(-t/\tau_1)]$$

$$+ (B - Q) * [1 - \exp(-t/\tau_2)]$$
$$+ S * [1 - \exp(-t/\tau_b)], \qquad (6)$$

where $\tau_b = 1/(\mu[293B]_o + \lambda)$, $y_\infty = \mu[293B]_o/(\mu[293B]_o + \lambda)$, $P = Ay_\infty/(1 - \tau_b/\tau_1)$, $Q = B\ y_\infty/(1 - \tau_b/\tau_2)$ and $S = P + Q - y_\infty/(A + B)$. Thus, the time course of I$_{Ks}$ in the presence of 293B could be fitted by the sum of three exponential functions.

Figure 5 shows an example of fitting equation. 6 to the I$_{Ks}$ recordings are $+60$ mV at different [293B]$_o$. First fitting the control of I$_{Ks}$ to equation. 3 yields values for τ_1 and τ_2 to 0.23 and 3.61 s, respectively. These values were then included in equation. 6 and I$_{Ks}$ in the presence of 293B fitted to the equation using the least-squares method. The values of τ_b, thus obtained form four cells, were shown in Figure 5B, where the
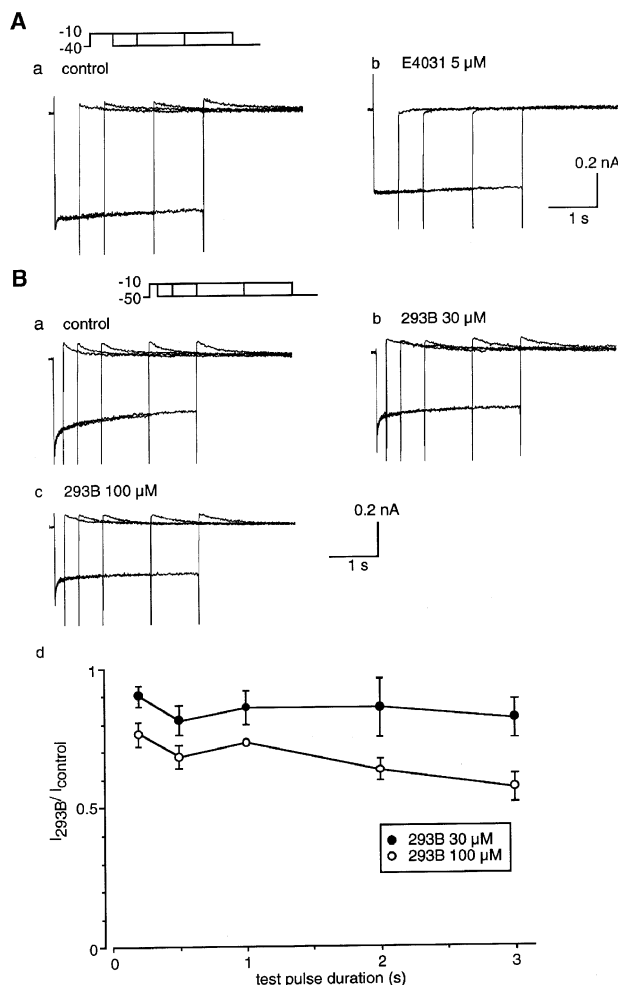

Figure 7 Effects of 293B on I$_{Kr}$. (A) An envelope test of tail currents. Superimposed current traces were obtained when the membrane was depolarized from -40 mV to -10 mV for various duration in the absence (a) and presence (b) of 5 μM E4031. The external solution is Ca^{2+}-free. (B) An envelope test of tail currents in the absence (a) and presence of 30 μM (b) 100 μM (c) 293B. The holding potential is -50 mV. (d) The amplitude of the tail current in the presence of 293B was normalized to that in the absence of drug and plotted against the pulse duration. The data are average of three experiments.

reciprocal of τ_b (τ_b^{-1}) was plotted against [293B]$_o$. At various test potentials a linear relation is evident, supporting 1 : 1 binding stoichiometry.

We then obtained the blocking (μ) and unblocking (λ) rate constants from the linear relation. The values of μ, λ and the ratio λ/μ were plotted at different membrane potentials (Figure 6). The blocking rate constant, μ, increased with membrane depolarization, and the regression line had a slope of an e-fold increase per 80.9 mV. On the other hand, the unblocking rate, λ, seemed to be constant at different membrane potentials. As a result, the ratio λ/μ was almost constant at potentials more positive than $+40$ mV (Figure 6C).

Effect of 293B on I$_{Kr}$

In the absence of E4031, 100 μM 293B inhibited the time-dependent outward current in response to depolarizing pulse and only small tail currents were observed upon relatively short pulse durations (Figure 1). Furthermore, the tail current was almost completely abolished during the prolonged depolarization (Figure 1). The findings may indicate that I$_{Kr}$ is also blocked

by 293B in a time-dependent manner. This property is particularly important when 293B is used as a tool to elucidate the physiological role of I_{Ks} in cardiac myocytes. Therefore the experiments were designed to examine whether 293B blocks I_{Kr} or not. It is known that in Ca^{2+}-free external solutions activation of I_{Kr} is shifted to more negative potentials and I_{Ks} activation moved more positive potentials (Sanguinetti & Jurkiewicz, 1992). Under these conditions current evoked by a depolarizing pulse to -10 mV from -40 or -50 mV seems predominantly I_{Kr}. This is demonstrated in Figure 7A, where the membrane was depolarized to -10 mV from -40 mV in the Ca^{2+}-free external solution, and current recordings with different pulse durations were superimposed. In control (a), the current shifted initially to downward direction upon depolarization, probably due to the lack of the inward rectifier K^+ current at -10 mV, and thereafter activation of I_{Kr} was observed. The tail current at repolarization was almost constant irrespective of different pulse durations in control (Aa), and was completely abolished by 5 μM E4031 (Ab). On the other hand, application of 30 μM (Bb) and 100 μM (Bc) 293B affected the tail current, although only slightly, in a concentration dependent manner. It should be noticed that the outward current during the depolarization was also depressed in a time-dependent manner by 293B. The amplitude of the tail current in the presence of 293B was normalized to that in control and plotted in Figure 7Bd. These findings indicate that high concentrations of 293B inhibit, although only slightly, I_{Kr} in a time-dependent manner.

Discussion

In response to a depolarizing pulse, the initial activation time course of I_{Ks} was overlapped irrespective of whether 293B exists or not, and thereafter the block occurred in a time-dependent manner. The extent of the block becomes larger and the rate of the block faster as $[293B]_o$ increases. Upon repolarization to -40 mV after preceding depolarization, i_{293B}/i_{cont} decreased transiently at moderate concentrations of 293B. The finding can be explained by assuming that the unblocking rate at the holding potential is relatively faster than the deactivation rate of I_{Ks}, i.e., the rapid unblock of the channel transiently increases the fraction of the open conformation upon repolarization. Thus, the findings obtained in the present study are consistent with a view that 293B is an open channel blocker for I_{Ks} (Loussouarn *et al.*, 1997). Alternatively, 293B may bind to a certain site of the channel in a state-dependent manner, i.e., 293B binds to the channel with the open conformation and unbinds if the channel is in the closed conformation.

I_{Ks} is known to consist of at least two different subunits, i.e., KvLQT1 channels and IsK regulator (Barhanin *et al.*, 1996; Sanguinetti, *et al.*, 1996). It has veen recently shown that 293B is a potent blocker of human KvLQT1 channels and binds to the channel protein itself but not to the IsK regulator (Loussouarn *et al.*, 1997). In COS-7 cells expressing KvLQT1 alone (without IsK), 293B blocked KvLQT1 current in both voltage- and time-dependent manner (Loussouarn *et al.*, 1997). In the present study, however, the IC_{50} values did not exert clear voltage-dependency over the voltage range from $+20$ to $+80$ mV. The difference is, in part, due to the voltage range examined. In the case of KvLQT1, the voltage-dependency of IC_{50} values was detected at potentials more negative than 0 mV (Loussouarn *et al.*, 1997), whereas we could not measure the $[293B]_o$-I_{Ks} block relationship at such negative potentials because the current size is very small in native I_{Ks}. In addition, it is well known that coexpression of IsK regulator with

KvLQT1 slows activation kinetics of KvLQT1 (Barhanin *et al.*, 1996; Sanguinetti *et al.*, 1996; Romey *et al.*, 1997). It is thus speculated that IsK regulator may alter the binding- or unbinding-rate of 293B, thereby blunting the voltage-dependency for I_{Ks}. In fact, Loussouarn *et al.* (1997) showed that 293B block takes a few tenths of ms in the absence of IsK and a few hundredths of ms in the presence of IsK.

On the other hand, the model calculation showed that the blocking rate increased with depolarization. The voltage-dependent block can be described by the following equation (Woodhull, 1973),

$$\mu = \mu_0 \exp(\delta z FE/RT) \tag{7}$$

Where μ_0 indicates the blocking rate at 0 mV, and z, F, E, R and T have their usual meanings in thermodynamics. When the above equation was applied to the voltage-dependency of the blocking rate (Figure 7), the values of μ_0 and δz were obtained to be 1.44×10^4 M^{-1} s^{-1} and 0.30. These values are relatively smaller in comparison to the rate constants for the ion block of other ionic channels in previous studies. For example, the rates of Mg^{2+} block of the ATP-sensitive K^+ channel and the inward rectifier K^+ channel are reported to be $1.5-2.0 \times 10^7$ M^{-1} s^{-1} (Horie *et al.*, 1987) and 1.1×10^5 M^{-1} s^{-1}, (Ishihara *et al.*, 1989) respectively. The rate constant for the Cs^+ and Rb^+ block of the inward rectifier K^+ current are 4×10^6 and 10^6 M^{-1} s^{-1} (Matsuda *et al.*, 1989) respectively. On the other hand, much slower rate constant has been reported for the Sr^{2+} block of the hyperpolarization-activated cation channel, i.e., the rate is 6.4 M^{-1} s^{-1} (Ono *et al.*, 1994).

To determine the blocking- and unblocking-rate, we used a mathematical model with an assumption that the fraction of the closed channels does not change after the addition of the blocker. Using an essentially similar calculation, Ono *et al.* (1994) characterized the time- and voltage-dependent block by Sr^{2+} of the hyperpolarization-activated cation current in rabbit sinoatrial node cells. They showed that the observed values such as blocking rate and dissociation constant were in agreement with those obtained by the concentration-jump method (Ono *et al.*, 1994). It should be noticed, however, that the above assumption may lead to under- or over-estimation of the block kinetics. At strong depolarization where the probability of channels staying in the closed states is negligibly small, the measurement of the rate constants might not be seriously affected. At moderate depolarization, however, the open probability is less than unity, and therefore the fraction of the closed channels should be affected by the presence of a blocked state. It should also be noted that the control I_{Ks} was fitted with the sum of two exponential functions in the present study. This fit does not necessarily represent the whole time course of I_{Ks}. This is because the onset of I_{Ks} is sigmoidal (Matsuura *et al.*, 1987; Balser *et al.*, 1990) and more than three closed states are considered to be present in the conformation of I_{Ks} channels (Balser *et al.*, 1990). Thus, the measurement of block kinetics is still preliminary in the present study.

In the experiment designed to examine the effect of 293B on I_{Kr} (Figure 7) the tail current was depressed only slightly but in a time-dependent manner. The inhibition was observed at >30 μM. The finding might be in good agreement with a previous finding that 30 μM 293B exerted no significant effects on I_{Kr} of guinea-pig hearts during a 500 ms depolarizing pulse (Busch *et al.*, 1997; Bosch *et al.*, 1998). However, in the absence of E4031 and presence of 100 μM 293B, the tail current could be recorded only upon relatively short pulse durations, and was completely abolished during prolonged depolarization (Figure 1). The finding can not be explained unless 293B blocks I_{Kr} in a time-dependent manner. It may be speculated

that 293B blocks I_{Kr} both in time- and voltage-dependent manner, i.e., depolarization to -10 mV inhibits the tail current slightly (Figure 7) but strong depolarization to $+60$ mV blocks I_{Kr} completely during the prolonged pulses as like that seen in Figure 1. In the present study, however, detailed measurement of block kinetics for I_{Kr} or use-dependency could not be carried out at various membrane potentials because the separation of I_{Kr} and I_{Ks} is difficult in native myocytes, particularly at positive potentials. Further study with a help of molecular biology is required to elucidate this point. Nonetheless, it seems that much higher concentrations of 293B are required to completely block I_{Kr} than those for I_{Ks} block. In addition, because of the slow kinetics the blocking effect of 293B on I_{Kr} might be negligible under the physiological condition where the action potential duration is usually less than a few hundredths of ms.

In conclusion, the present results indicate that 293B blocks I_{Ks} in native myocytes in a time-dependent manner. The blocking rate, measured by using a mathematical model, is relatively slower than those reported for open channel blockage of various ionic channels reported in previous studies.

References

BARHANIN, J., LESAGE, F., GUILLEMARE, E., FINK, M., LAZDUNSKI, M. & ROMEY, G. (1996). KvLQT1 and IsK (minK) proteins associate to form the I_{Ks} cardiac potassium current. *Nature,* **384,** 78 – 89.

BALSER, J.R., BENNET, P.B. & RODEN, D.M. (1990). Time-dependent outward current in guinea-pig ventricular myocytes: Gating kinetics of the delayed rectifier. *J. Gen. Physiol.,* **96,** 835 – 863.

BOSCH, R.F., GASPO, R., BUSCH, A.E., LANG, H.J., LI, G.R. & NATTEL, S. (1998). Effects of the chromanol 293B, a selective blocker of the of the slow component of the delayed rectifier K^+ current, on repolarization in human and guinea pig ventricular myocytes. *Cardiovasc. Res.,* **38,** 441 – 450.

BUSCH, A.E., SUESSBRICH, H., WALDEGGER, S., SAILER, E., GREGER, R., LANG, H.-J., LANG, F., GIBSON, K. & MAYLIE, J.G. (1996). Inhibition of I_{Ks} in guinea pig cardiac myocytes and guinea pig I_{sK} channels by the chromanol 293B. *Pflügers Arch.,* **432,** 1094 – 1096.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.,* **391,** 85 – 100.

HORIE, M., IRISAWA, H. & NOMA, A. (1987). Voltage-dependent magnesium block of adenosine-triphosphate-sensitive potassium channel in guinea-pig ventricular cells. *J. Physiol.,* **387,** 251 – 271.

ISENBERG, G. & KLÖCKNER, U. (1982). Calcium tolerant ventricular myocytes prepared by preincubation in a 'KB Medium'. *Pflügers Arch.,* **395,** 6 – 18.

ISHIHARA, K., MITSUIYE, T., NOMA, A. & TAKANO, M. (1989). The Mg^{2+} block and intrinsic gating underlying inward rectification of the K^+ current in guinea-pig cardiac myocytes. *J. Physiol.,* **419,** 297 – 320.

LOUSSOUARN, G., CHARPENTIER, F., MOHAMMAD-PANAH, R., KUNZELMANN, K., BARO, I. & ESCANDE, D. (1997). KvLQT1 potassium channel but not IsK is the molecular target for trans-6-cyano-4-(N-ethylsulfonyl-N-methylamino)-3-hydroxy-2,2-dimethyl-chromane. *Mol. Pharmacol.,* **52,** 1131 – 1136.

MATSUDA, H., MATSUURA, H. & NOMA, A. (1989). Triple-barrel structure of inwardly rectifying K^+ channels revealed by Cs^+ and Rb^+ block in guinea-pig heart cells. *J. Physiol.,* **413,** 139 – 157.

MATSUURA, H., EHARA, T. & IMOTO, Y. (1987). An analysis of the delayed outward current in single ventricular cells of the guinea-pig. *Pflügers Arch.,* **410,** 596 – 603.

ONO, K., MARUOKA, F. & NOMA, A. (1994). Voltage- and time-dependent block of I(f) by Sr^{2+} in rabbit sino-atrial node cells. *Pflügers Arch.,* **427,** 437 – 443.

RODEN, D.M. (1993). Current status of class III antiarrhythmic drug therapy. *Am. J. Cardiol.,* **72,** 44B – 49B.

ROMEY, G., ATTALI, B., CHOUABE, C., ABITBOL, I., GUILLEMARE, E., BARHANIN, J. & LAZDUNSKI, M. (1997). Molecular mechanism and functional significance of the MinK control of the KvLQT1 channel activity. *J. Biol. Chem.,* **272,** 16713 – 16716.

SANGUINETTI, M.C., CURRAN, M.E., SPECTOR, P.S., ZOU, A., SHEN, J., ATKINSON, D. & KEATING, M.T. (1996). Coassembly of KvLQT1 and minK (IsK) to form cardiac I_{Ks} potassium channel. *Nature,* **384,** 80 – 83.

SANGUINETTI, M.C. & JURKIEWICZ, N.K. (1990). Two components of cardiac delayed rectifier K^+ current. *J. Gen. Physiol.,* **347,** 641 – 657.

SANGUINETTI, M.C. & JURKIEWICZ, N.K. (1992). Role of external Ca^{2+} and K^+ in gating of cardiac delayed rectifier K^+ currents. *Pflügers Arch.,* **420,** 180 – 186.

SHINBO, A. & IIJIMA, T. (1997). Potentiation by nitric oxide of the ATP-sensitive K^+ current induced by K^+ channel openers in guinea-pig ventricular cells. *Br. J. Pharmacol.,* **120,** 1568 – 1574.

SPECTOR, P.S., CURRAN, M.E., KEATING, M.T. & SANGUINETTI, M.C. (1996). Class III antiarrhythmic drugs block HERG, a human cardiac delayed rectifier K^+ channel. *Circ. Res.,* **78,** 499 – 503.

WOODHULL, A.M. (1973). Ionic blockage of sodium channels in nerve. *J. Gen. Physiol.,* **61,** 683 – 708.