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Inhibitory Actions of HERG Currents by the Immunosuppressant Drug Cyclosporin A

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The effect of cyclosporin A (CsA), an immunosuppressant, on human ether-a-go-go-related gene (HERG) channel as it is expressed in human embryonic kidney cells was studied using a whole-cell, patch-clamp technique. CsA inhibited the HERG channel in a concentration-dependent manner, with an IC_{50} value and a Hill coefficient of 3.17 μM and 0.89, respectively. Pretreatment with cypermethrine, a calcineurin inhibitor, had no effect on the CsA-induced inhibition of the HERG channel. The CsA-induced inhibition of HERG channels was voltage-dependent, with a steep increase over the voltage range of the channel opening. However, the inhibition exhibited voltage independence over the voltage range of fully activated channels. CsA blocked the HERG channels predominantly in the open and inactivated states rather than in the closed state. Results of the present study suggest that CsA acts directly on the HERG channel as an open-channel blocker, and it acts independently of its effect on calcineurin activity.

Key Words: Cyclosporin A, Immunosuppressant, HERG, Long QT syndrome, Open channel block

INTRODUCTION

Cyclosporin A (CsA) is a potent immunosuppressant widely used to prevent the incidence of organ transplant rejection and to treat autoimmune diseases [1]. Several immunosuppressive mechanisms of CsA have been reported. CsA binds to cyclophilin via intracellular receptors for CsA that are largely expressed in the nervous system, and the complex of CsA-cyclophilin inhibits calcineurin, a Ca^{2+}-calmodulin-dependent phosphatase 2B, which results in preventing calcineurin-dependent interleukin-2 transcription and T-cell activation [2,3]. Therefore, the extent of calcineurin inhibition is correlated with immunosuppressive activity. However, chronic treatment with CsA is restricted by serious side effects such as nephrotoxicity, cardiotoxicity, hyperkalemia, and neurotoxicity [4]. Although the causes of CsA-induced side effects are not fully understood, they may be independent of immunosuppressive activities. For example, CsA caused spontaneous- or stimulation-induced epileptiform activity, thus affecting neuronal excitability in a hippocampal slice [5,6]. In addition, CsA induces a time-dependent and rapid membrane depolarization in T-lymphocytes [7,8]. These results raised the possibility that CsA may modulate the activity of ion channels and receptors that play major roles in regulating neuronal excitability. Several recent studies have shown that CsA inhibits the voltage-gated K^+ channel in human lymphocytes, resulting in a membrane depolarization [9], and it inhibits the voltage-gated Ca^{2+} channel (VGCC) in cultured hippocampal neurons, resulting in inhibition of VGCC-dependent long-term potentiation [10]. These results indicate that CsA is likely to affect channel activity, although it is not clear whether the action of CsA on ion channels is mediated by a calcineurin-dependent or -independent mechanism.

In ventricular myocytes of the heart, reduced function of the cardiac K^+ current (I_K) causes long QT syndrome. The I_K are the rapidly (I_{Kr}) and slowly (I_{Ks}) activating components of the delayed rectifier K^+ current [11,12]. The I_{Kr} is encoded by the human ether-a-go-go-related gene (HERG), and muta-

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ABBREVIATIONS: HERG, human ether-a-go-go-related gene; HEK-293, human embryonic kidney; SSRIs, selective serotonin reuptake inhibitors; CsA, cyclosporin A; I_K, cardiac K^+ current; I_{Kr}, rapidly activating components of the delayed rectifier K^+ current; I_{Ks}, slowly activating components of the delayed rectifier K^+ current; LQT2, long QT syndrome; MEM, minimum essential medium.

tion in the HERG has been shown to cause chromosome 7-linked inherited long QT syndrome (LQT2), which is related to ventricular tachyarrhythmia Torsades de pointes, and, potentially, to sudden death [13,14]. Several drugs that block HERG channels have been shown to cause cardiotoxicity [15-17].

In the present study, we examined the effects of CsA on HERG channels stably expressed in human embryonic kidney (HEK293) cells, and characterized the nature of the action.

METHODS

Cell culture and transfection

HEK293 cells stably expressing HERG channels, a kind gift from Dr C. January [18], were used for electrophysiological recordings. The method for establishing HERG channels expression in HEK293 cells is briefly described as follows. HERG cDNA was transferred into the plasmid expression vector pCDNA3 vector (Invitrogen Corporation, San Diego, CA, USA). HEK293 cells were stably transfected with HERG cDNA using either a calcium phosphate precipitate method (Invitrogen) or a lipofectamine method (Invitrogen). The transfected cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum: 1 mM sodium pyruvate, 0.1 mM non-essential amino-acid solution, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate. The cultures were passaged every 4~5 days with a brief trypsin-EDTA treatment followed by seeding onto glass coverslips (diameter: 12 mm, Fisher Scientific, Pittsburgh, PA, USA) in a Petri dish. After 12~24 h, the cell-attached coverslips were used for electrophysiological recordings.

Electrophysiological recordings

The HERG currents of HEK293 cells were recorded using a whole-cell, patch-clamp technique [19] at room temperature (22~23°C). The micropipettes fabricated from glass capillary tubing (PG10165-4; World Precision Instruments, Sarasota, FL, USA) with a double-stage vertical puller (PC-10; Narishige, Tokyo) had a tip resistance of 2~3 MΩ when filled with the pipette solution. Whole-cell currents were amplified using an Axopatch 1 D amplifier (Molecular Devices, Sunnyvale, CA, USA) digitized with Digidata 1,200 A (Molecular Devices) at 5 kHz and low-pass filtered with a four-pole Bessel filter at 2 kHz. Capacitive currents were canceled and series resistance was compensated at 80% with the amplifier, while leak subtraction was not used. The generation of voltage commands and acquisition of data were controlled with pClamp 6.05 software (Molecular Devices) running on an IBM-compatible Pentium computer. The recording chamber (RC-13, Warner Instrument Corporation, Hamden, CT, USA) was continuously perfused with bath solution (see below for composition) at a rate of 1 ml/min.

Solutions and drugs

The external solution contained 137 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES (adjusted to pH 7.4 with NaOH). The intracellular solution contained 130 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA, 5 mM MgATP, and 10 mM HEPES (adjusted to pH 7.4 with KOH). CsA and cypermethrine (Sigma Chemical Co., St. Louis, MO, USA) were dissolved in dimethyl sulfoxide (DMSO) at 30 mM and 50 mM, respectively, and were further diluted into the bath solution. The final concentration of DMSO in the bath solution, which was less than 0.1%, had no effect on HERG currents (Data not shown).

Data analysis

Data were analyzed with Origin 7.0 (OriginLab Corp., Northampton, MA, USA) and Clampfit 6.05 software (Molecular Devices). An IC_{50} and Hill coefficient (n) were obtained by fitting concentration dependence data to the following equation:

$$I(\%)=1/\{1+(IC_{50}/[D])^{n}\} \quad (1)$$

in which I (%) is the percent inhibition of current ($I(\%)=[1-I_{drug}/I_{control}]\times100$) at test potential and [D] represents various drug concentrations. The steady-state activation/inactivation curves were fitted with the Boltzmann equation:

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

where k represents the slope factor, V the test potential, and $V_{1/2}$ the potential at which the conductance was half-maximal.

Results were expressed as mean±standard error of the mean (SEM). Paired or unpaired Student's t-test and analysis of variance (ANOVA) were used for statistical analysis. A value of $p<0.05$ was considered statistically significant.

RESULTS

Inhibition of HERG-mediated current (I_{HERG}) by CsA

We examined the effects of CsA on I_{HERG}, using a whole-cell, patch-clamp technique. As shown in Fig. 1A (upper trace), whole-cell currents were elicited with 4-s depolarization to +20 mV from a holding potential of −80 mV, and the tail current was recorded at −60 mV for 6 s in HEK293 cells expressing HERG channels. Bath-applied CsA reduced the I_{HERG} in a concentration-dependent manner (Fig. 1A). As shown in Fig. 1B, dose dependency of the steady-state currents measured at the end-pulse of +20 mV or at peak tail currents was analyzed quantitatively. A nonlinear least-squares fit of dose-response plots with the Hill equation yielded an IC_{50} value of 3.77±0.48 μM and a Hill coefficient of 0.92±0.11 (n=7) for the steady-state currents, and an IC_{50} value of 3.17±0.29 μM and a Hill coefficient of 0.87±0.08 (n=7) for the peak tail currents.

Effect of calcineurin inhibitor on CsA-induced inhibition of I_{HERG}

The HERG channel can be phosphorylated and dephosphorylated [20,21], and CsA has been classified as a phosphatase inhibitor. To elucidate whether calcineurin is involved in the CsA-induced inhibition of I_{HERG}, we further investigated the effect of cypermethrine, a calcineurin inhibitor, in bath solution. Fig. 2A shows the effects of cypermethrine on the inhibition of HERG by CsA. A 5-min

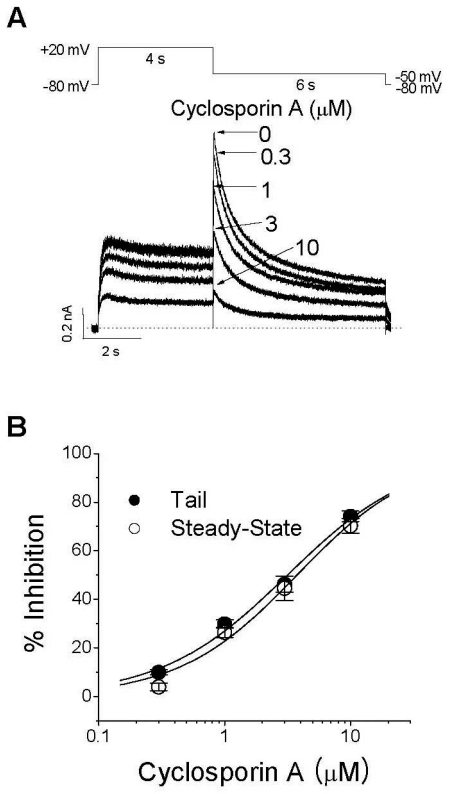

Fig. 1. Concentration dependence of CsA-induced inhibition of I_{HERG}. (A) Superimposed I_{HERG} traces were elicited with 4-s depolarization to +20 mV from a holding potential of −80 mV, and the tail current was recorded at −60 mV for 6 s in the absence and presence of 0.3, 1, 3, and 10 μM CsA, as indicated. The protocol was applied every 15 s. The dotted line represents zero current. (B) Concentration-dependent curve of inhibition by CsA for steady-state currents (open circle) measured at the end of a depolarizing pulse to +20 mV or peak tail currents (closed circle). The respective percentage inhibitions were plotted against various concentrations of CsA. The solid lines are fitted to the data points by the Hill equation. Data are expressed as mean±SEM.

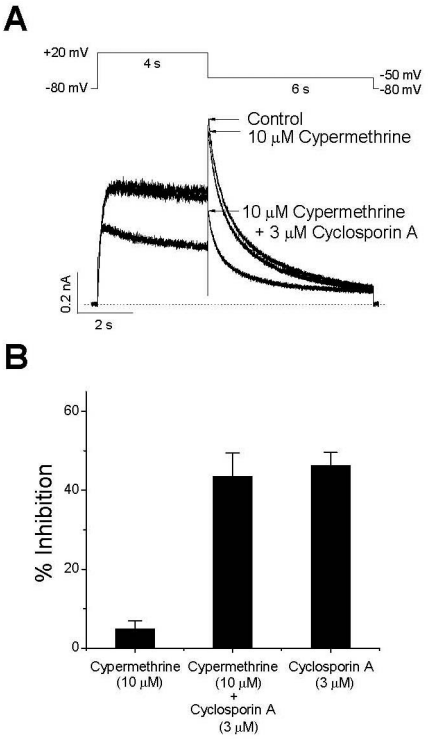

Fig. 2. The effects of a calcineurin inhibitor on the inhibition of I_{HERG} by CsA. Representative superimposed currents were elicited with 4-s depolarization to +20 mV from a holding potential of −80 mV, and the tail current was recorded at −60 mV for 6 s every 15 s. (A) Control current, the current recorded after 5-min exposure to 10 μM cypermethrine, and the current measured after a further 3-min treatment with 3 μM CsA are shown. The dotted line represents zero current. (B) Peak tail currents under a set of experimental conditions (A) were normalized to those of the control, and are displayed as a percentage of inhibition to show the effects of 10 μM cypermethrine (n=5), 10 μM cypermethrine with 3 μM CsA (n=5), and 3 μM CsA (n=7, Fig. 1). Data are expressed as mean±SEM.

exposure to 10 μM cypermethrine had little effect on I_{HERG} (less than ~5% inhibition). After a 5-min exposure to 10 μM cypermethrine, 3 μM CsA inhibited the peak tail currents of I_{HERG} by 43.53±5.95% inhibition (n=5), which was not significantly different from the inhibition induced by CsA (46.26±3.32% inhibition, n=5) in the absence of cypermethrine (Fig. 1B, 2B). The lack of effects of cypermethrine on CsA-induced inhibition of I_{HERG} strongly indicates that CsA directly inhibits I_{HERG} in a calcineurin-independent manner.

Voltage dependency of CsA-mediated inhibition of I_{HERG}

We next investigated the I_{HERG}-voltage (I-V) relationship (Fig. 3). I_{HERG} was produced by applying 4-s depolarizing pulses between −70 and +60 mV in 10-mV increments every 15 s from a holding potential of −80 mV. The tail current was recorded at −60 mV for 6 s. Fig. 3A shows representative superimposed current traces recorded under control conditions and 3 min after exposure to 3 μM CsA in the same cell. The normalized I-V relationships for I_{HERG} measured at the end of the depolarizing pulses and for the peak tail current are shown in Fig. 3B and C, respectively. Under control conditions, the I_{HERG} measured at the end of the depolarizing pulses were generated at −40 mV, peaked at about 0 mV, and decreased thereafter, which resulted in a negative slope of the I-V curve, while the tail currents were fully activated following pulses from positive to 0 mV. In the presence of 3 μM CsA, I_{HERG} measured at the end of the depolarizing pulses and tail current amplitudes were reduced through the entire range of voltages, for which the HERG channel was activated. To examine the effect of CsA on voltage-dependent activation of HERG channels, the tail peak currents were normalized and plotted against the membrane potential (Fig. 3C). Data were fitted with a Boltzmann function. The activation curve was unchanged in the presence of 3 μM CsA. The potential at half-maximum activation ($V_{1/2}$) was −19.40±2.13 mV and −21.55±1.92 mV before and after application of the drug (n=5), respectively. Similarly, there was no significant shift in slope value (k) in the presence of CsA (5.95±0.67 mV for control; 7.39±0.50 mV for CsA, n=5).

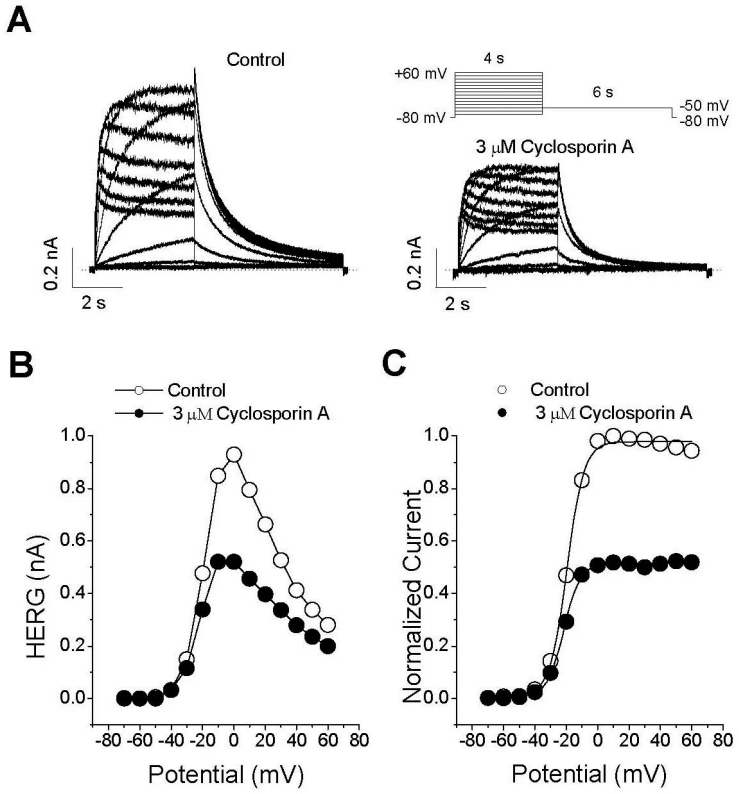

Fig. 3. Effect of CsA on the I_{HERG}-voltage (I-V) relationship. (A) Representative superimposed current traces recorded under control conditions and 3-min exposure to 3 μM CsA. I_{HERG} was produced by applying 4-s depolarizing pulses between −70 and +60 mV in 10-mV increments every 15 s from a holding potential of −80 mV. Tail current was recorded at −60 mV for 6 s. The dotted lines represent zero current. (B) The I-V relationships for I_{HERG} were measured at the end of the depolarizing pulses and for peak tail current. (C) Normalized activation curves were recorded under control conditions and 3 min after exposure to 3 μM CsA. The solid lines represent the activation curves obtained by normalization to the tail peak amplitude from (A) and by fitting those data to the Boltzmann equation (see *METHODS*, equation 2).

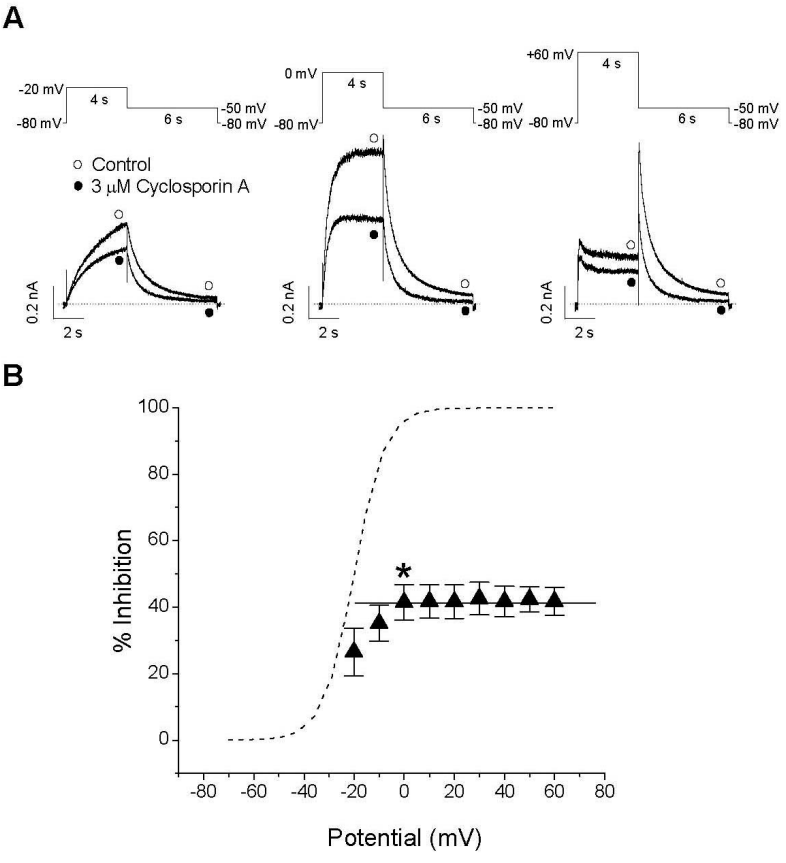

Fig. 4. Voltage dependence of CsA-induced inhibition of I_{HERG}. (A) Representative superimposed current traces under control conditions and in the presence 3 μM CsA selected at three different potentials (−20, 0 and +60 mV) recorded using the pulse protocols. The dotted lines represent zero current. (B) Percentage of current inhibition (closed triangle) at different membrane potentials. The tail currents in the presence of 3 μM CsA were normalized to the tail current obtained under control conditions. The dashed line represents the activation curve of the HERG channel under control conditions, which was calculated by measuring tail current amplitudes and by fitting those data to the Boltzmann equation (see *METHODS*, equation 2). For potentials positive to 0 mV, the solid line was drawn from a linear curve fitting (n=5; *$p<0.05$ versus data at −20 mV). Data are expressed as mean±SEM.

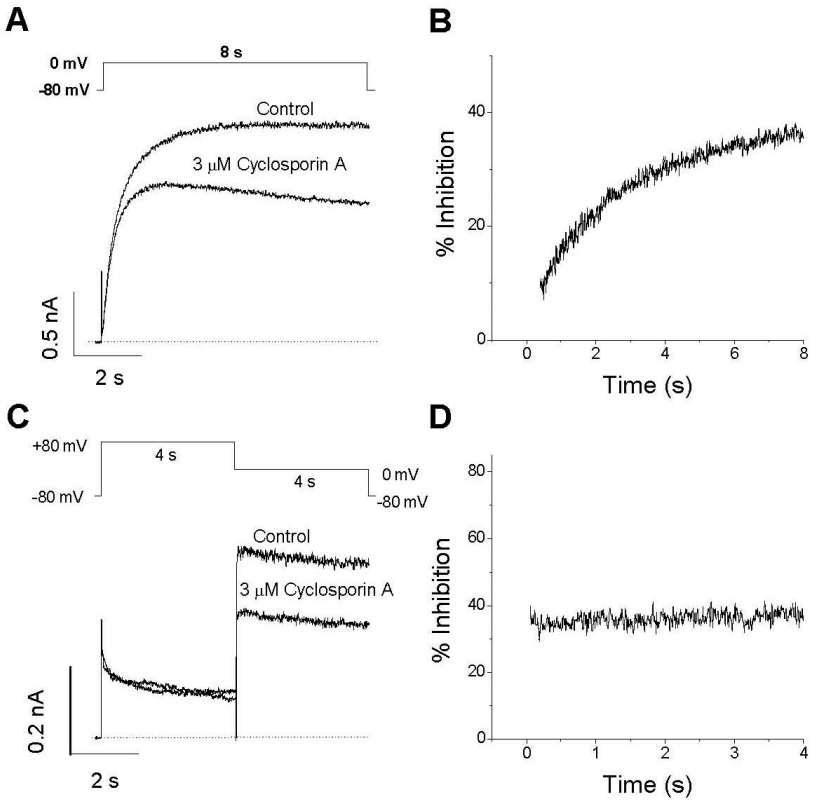

Fig. 5. State-dependent inhibition of I_{HERG} by CsA. (A) Representative superimposed current traces under control conditions and after application of 3 μM CsA for 3 min. Cells were held at a holding potential of −80 mV to the channels in the closed state before a single depolarizing step to 0 mV for 8 s resumed in the presence of CsA. (B) The % inhibition was obtained by division and plotted as a function of time. A strong time-dependent development of I_{HERG} inhibition by CsA was detected, suggesting an open channel block by CsA. (C) Superimposed typical current traces under control conditions and after application of 3 μM CsA for 3 min with a holding potential of −80 mV, which were obtained by a first 4-s depolarizing pulse of +80 mV followed by a second 4-s depolarizing pulse of 0 mV. (D) The normalized fractional inhibition. The % inhibition upon channel opening during the second voltage step (0 mV) was obtained by division and plotted against time. Maximum inhibition was detected in the inactivated state during the first step pulse, and no further time-dependent inhibition occurred upon channel opening during the second voltage step. The dotted lines represent zero current.

To examine the voltage dependence of the CsA effect, we compared the extent of percent inhibition of I_{HERG} (see *METHODS*) at different potentials. Fig. 4A shows representative superimposed current traces under control conditions and in the presence of 3 μM CsA selected at three different potentials. Fig. 4B shows the relative % inhibition of I_{HERG} against membrane potential. A high degree of inhibition with a strong voltage dependence was observed between −20 and 0 mV, which involved the voltage range of the HERG channel opening (Fig. 4B). However, the inhibition of I_{HERG} by CsA at potentials between 0 and +60 mV, where the channels were fully activated, lacked such a voltage dependence: 41.42±5.25% inhibition value at 0 mV and 41.73±4.19% inhibition at +60 mV (n=5, ANOVA, $p<0.05$). The linear curve fitting of the data at positive potentials (Fig. 4B, solid line) showed a slope of zero. In sum, the voltage-dependence of the effect of CsA implies that the inhibition of I_{HERG} occurs preferentially after the channels are open. Furthermore, the data indicate that, once channels are fully activated, the effect of CsA is independent of membrane potentials.

State-dependence of the CsA-mediated inhibition of I_{HERG}

To examine the state-dependence (closed or activated, i.e., open and/or inactivated) of channel blockage, we activated currents under control conditions and in the presence of the drug using a single depolarizing step to 0 mV for 8 s (Fig. 5A). Having obtaining a control measurement, 3 μM CsA was applied to the cell for 3 min to allow an equilibration of drug concentrations within the bath and the cell. For this experiment, cells were held continuously at −80 mV to the channels in a closed state. Then the depolarization protocols were resumed. Fig. 5B shows the time course of inhibition by plotting the percent inhibition of I_{HERG} (see *METHODS*) against time. A time-dependent development of I_{HERG} inhibition by CsA was detected (37.3±3.8% inhibition at the end of the 0 mV depolarizing step, n=5). This showed that I_{HERG} was only slightly blocked by CsA when the HERG channels were predominantly in the closed state. Therefore, the block of I_{HERG} by CsA requires channel activation from the closed state.

In order to examine whether HERG channels in their inactivated state are blocked by CsA, a long test pulse to +80 mV for 4 s was applied to inactivate the channels, which was followed by a second voltage step of 0 mV for 4 s to open the HERG channels. Fig. 5C shows representative current traces under control conditions and after a 3-min treatment with 3 μM CsA while holding the cell at −80 mV. As shown in Fig. 5D, the normalized fractional inhibition upon channel opening during the second voltage step (0 mV) was not time-dependent, indicating no additional inhibition while the channels were re-activated (38.2±4.1% inhibition at the end of the 0 mV depolarizing step, n=5). This result suggests that the pronounced inhibition of the I_{HERG} had already been reached during the previous inactivating 80-mV pulse. On the basis of the above overall data (Fig. 5), it can be concluded that CsA inhibits the HERG channels predominantly in the open and inactivated state rather than in the closed state.

DISCUSSION

In the present study, we found that CsA inhibited cloned HERG channels expressed in a human cell line. CsA has been classified as a phosphatase inhibitor of calcineurin. The immunosuppressive mechanism of CsA is believed to be mediated by a calcineurin-dependent mechanism [2,3]. For example, CsA inhibits calcineurin, a Ca^{2+}-calmodulin-dependent phosphatase 2B, resulting in the prevention of calcineurin-dependent interleukin-2 transcription and T-cell activation. Therefore, the extent of calcineurin inhibition is highly correlated with immunosuppressive activity, and the HERG channel could be modulated by serine/threonine kinase and tyrosine kinase via phosphorylation or dephosphorylation [20,21]. However, the present study shows that a calcineurin inhibitor, cypermethrine did not prevent the CsA-induced block of I_{HERG} (Fig. 2). This result strongly suggests that CsA directly blocks I_{HERG} in a calcineurin-independent manner. Additionally, the present study was performed in whole-cell recordings, during which Ca^{2+} was buffered by EGTA to a resting concentration (less than 10 nM). Because the catalytic activity of calcineurin requires a significant increase of intracellular free Ca^{2+}, the present experiment conditions suggest that the CsA-induced block of I_{HERG} is independent of calcineurin activity.

The characteristics of the CsA-induced block of I_{HERG} were state-dependent. First, a time-dependent development of I_{HERG} inhibition by CsA was detected (Fig. 5A, B). This result suggests that CsA is not a blocking factor when the HERG channels are predominantly in the closed state and that a CsA-induced block of I_{HERG} requires channel activation from the closed state. Second, a CsA-induced block did not occur upon re-activation of the channel with a 0-mV pulse after the occupancy of the inactivated state of the channel had been maximal at a +80-mV pulse (Fig. 5C, D). This result suggests that the maximal block of the I_{HERG} had already been reached during the previous inactivating +80-mV pulse. Third, a strong voltage-dependent block of I_{HERG} by CsA was observed between −20 and 0 mV in the voltage ranges of the HERG channel opening (Fig. 4). The above summarized results suggest that CsA blocks the HERG channels predominantly in the open and inactivated state rather than in the closed state.

In open-channel blocking mechanisms, a strong voltage dependence has been detected in the voltage range of the channel opening [22,23]. However, in the voltage range where the HERG channel is fully activated, an additional voltage dependence may be governed by the charged or uncharged form of the drug. In the present study, the CsA-induced block of the I_{HERG} was voltage-independent of the voltage range at which channels are fully activated (Fig. 4). CsA is predominately in an uncharged neutral form at physiological pH [1], resulting in independence of the transmembrane electric field regardless of whether a drug accesses its binding site on the HERG channel from the intracellular or the extracellular surface. Under the present experiment conditions, CsA was mainly in the uncharged form at the intracellular pH of 7.3 (pH of the pipette solution). Therefore, the block of I_{HERG} by CsA in the range of voltages where the channels were fully activated, did not show voltage dependence.

The therapeutic plasma concentration of CsA in patients with transplantation is in the range of 0.1∼0.2 μM. In the present study, the IC_{50} value of CsA for blocking is around 3∼4 μM, which is much higher than therapeutic plasma concentrations. Therefore, at a clinical level, CsA may not induce cardiac disorder by the inhibition of I_{HERG}. However, in the present study, a small but significant reduction in I_{HERG} at concentrations as low as 0.3 μM CsA was detected, but these effects were examined in a HEK293 cell line. Thus, the phospholipid composition of the cell may be different from human native cardiac myocytes, and the differences in membrane composition may affect the CsA-induced I_{HERG} blockade. Furthermore, drug concentrations may be higher in tissues than in plasma due to a high lipophilicity and affinity for adipose tissues. Therefore, it is possible that in the present study, the extent of the blocking effects of CsA on I_{HERG} under physiological conditions may be underestimated. That is, the CsA-induced block of I_{HERG} could be clinically relevant in the upper range of therapeutic plasma concentrations. Therefore, it is most likely that the CsA-induced block of the HERG channel may cause cardiotoxicity via a pronged QT interval and subsequent ventricular tachyarrhythmia Torsades de pointes.

In conclusion, the present study is the first recorded evidence that CsA blocks the HERG channel via a novel calcineurin-independent pharmacological property, which indicates a direct blocking effect of CsA on cloned HERG channels.

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