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Direct Block of Cloned K^+ Channels, Kv1.5 and Kv1.3, by Cyclosporin A, Independent of Calcineurin Inhibition

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The interaction of cyclosporine A (CsA), an immunosuppressant, with rat brain Kv1.5 (Kv1.5) channels, which were stably expressed in Chinese hamster ovary cells, was investigated using the whole-cell patch-clamp technique. CsA reversibly blocked Kv1.5 currents at +50 mV in a reversible concentration-dependent manner with an apparent IC_{50} of 1.0 μM. Other calcineurin inhibitors (cypermethrin, autoinhibitory peptide) had no effect on Kv1.5 and did not prevent the inhibitory effect of CsA. Fast application of CsA led to a rapid and reversible block of Kv1.5, and the onset time constants of the CsA-induced block were decreased in a concentration-dependent manner. The CsA-induced block of Kv1.5 channels was voltage-dependent, with a steep increase over the voltage range of channel opening. However, the block exhibited voltage independence over the voltage range in which channels were fully activated. The rate constants for association and dissociation of CsA were 7.0 $\mu M^{-1}s^{-1}$ and 8.1 s^{-1}, respectively. CsA slowed the deactivation time course, resulting in a tail crossover phenomenon. Block of Kv1.5 by CsA was use-dependent. CsA also blocked Kv1.3 currents at +50 mV in a reversible concentration-dependent manner with an apparent IC_{50} of 1.1 μM. The same effects of CsA on Kv1.3 were also observed in excised inside-out patches when applied to the internal surface of the membrane. The present results suggest that CsA acts directly on Kv1.5 currents as an open-channel blocker, independently of the effects of CsA on calcineurin activity.

Key Words: Cyclosporine A, Kv1.5, Kv1.3, Calcineurin inhibitor, Open channel block

INTRODUCTION

CsA is a potent immunosuppressant widely used to prevent the incidence of organ transplant rejection and to treat various autoimmune disease (Sigal & Dumont, 1992). The cellular and molecular mechanism of the immunosuppressive effect of CsA is well known: CsA binds to cyclophilin, the intracellular receptors for CsA which are enriched in the nervous system, and the complex of CsA-cyclophilin inhibits calcineurin, a Ca^{2+}-calmodulin-dependent phosphatase 2B, subsequently preventing calcineurin-dependent interleukin-2 transcription and T-cell activation (Steiner et al, 1996; Matsuda & Koyasu, 2000). Thus, there is a close correlation between the degree of calcineurin inhibition and immunosuppressive activity.

Calcineurin is known to regulate secretion of glutamate and norepinephrine, and gating kinetics of glutamate receptors, voltage-gated Na^+ and Ca^{2+} channels (Yakel, 1997). CsA, a calcineurin inhibitor, inhibits voltage-gated Ca^{2+} channel (VGCC) in cultured hippocampal neurons, resulting in inhibition of VGCC-dependent long-term potentiation (Onuma et al, 1998). In addition, CsA significantly reduces the functional expression of Kir2.1 potassium channels and blocks the downregulation of Nav1.8 channels in *Xenopus* oocyte (Chen et al, 1998; Choi & Soderlund, 2004). In contrast, the inhibition of calcineurin with CsA increases expression of cell surface functional Na^+ channels in adrenal chromaffin cells (Shiraishi et al, 2001). These observations indicate that CsA is likely to affect channel indirectly via inhibition of the phosphatase activity of calcineurin. However, several studies have suggested a direct and nonspecific effect of CsA on membrane structure and function of different cells. For example, CsA elicited spontaneous- or stimulation-induced epileptiform activity, thus affecting the neuronal excitability in hippocampal slice (Tauboll et al, 1998; Wong & Yamada, 2000). One possible explanation is that CsA modulates the activity of several ion channels and receptors that play major roles in regulating neuronal excitability. In addition, CsA produces a time-dependent and rapid membrane depolarization in T-lymphocyte (Damjanovich et al, 1987; Tordai et al, 1992). These results also raised the possibility that CsA may modulate ion channel activity via mechanisms, not involving effects on calcineurin. Indeed, CsA reduces the voltage-gated K^+ current in human lymphocyte, resulting in a depolarized membrane potential (Panyi et al, 1996).

In the present study, therefore, we investigated the effects of CsA on cloned K^+ channel Kv1.5 and Kv1.3 to test

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ABBREVIATIONS: VGCC, voltage- gated Ca^{2+} channel; PCR, polymerase chain reaction.

the direct interaction between the drug and the channel.

METHODS

Stable transfection and cell culture

CHO cells (ATCC, Rockville, MD, USA) were maintained in Iscove's modified Dulbecco's medium (IMDM; Invitrogen Corporation, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 0.1 mM hypoxanthine and 0.01 mM thymidine. The CHO cells used were stably expressed Kv1.5 or Kv1.3 channels as previously described (Choi et al, 1999; Choi et al, 2000). Briefly, Kv1.3 and Kv1.5 cDNA clone were subcloned into expression vector pRc/CMV and pCR3.1 (Invitrogen Corporation, Grand Island, NY, USA), respectively using polymerase chain reaction (PCR). The plasmid DNAs containing the Kv1.3 and Kv1.5 cDNA were purified by a DNA purification kit (Promega, Madison, WI, USA). To produce transfection, CHO cells in IMDM were incubated with the Kv1.3 and Kv1.5 cDNA construct mixed with lipofectamine™ reagent (Invitrogen Corporation) and FuGENE™6 (Boehringer Mannheim, Indianapolis, IN, USA), respectively. The cells were incubated for 48 h under 95% humidified air-5% CO_2 environment at 37°C and subcultured by 1 : 10 dilution in IMDM containing 0.5 mg/ml G418 (Invitrogen Corporation). After 2 weeks, antibiotic-resistant clones were randomly selected and cultured in IMDM containing 0.2 mg/ml of G418. The medias were changed every 2~3 days with a fresh IMDM containing 0.2 mg/ml G418 and passed every 4~5 days by the use of a brief trypsin-EDTA treatment. The trypsin-EDTA treated cells were seeded onto glass coverslips (diameter: 12 mm, Fisher Scientific, Pittsburgh, PA, USA) in a Petri dish 24 h before use. For electrophysiological experiments, cells-attached coverslips were transferred to a continually perfused recording chamber (RC-13, Warner Instrument Corporation, Hamden, CT, USA).

Electrophysiological recordings

The currents were recorded at room temperature (22~24°C) using the whole-cell and inside-out configuration of the patch-clamp technique with an Axopatch 1D patch clamp amplifier (Axon Instruments, Union City, CA, USA). Micropipettes were pulled from PG10165-4 glass capillary tubing (World Precision Instruments, Sarasota, FL, USA) and had resistances of 2~3 MΩ when filled with internal pipette solution. Liquid junction potentials between external and pipette solution were offset before the pipette touched the cell. The micropipettes were gently lowered onto the cells, and gigaohm seal formation was achieved by suction. Seal resistances were in the range of 2~10 GΩ. Following pipette capacitance compensation, cells were ruptured with brief additional suction. Thereafter, whole-cell capacitative currents were compensated with analog compensation without leakage compensation. Sampling frequency was 5 kHz, and currents were filtered at 2 kHz (four-pole Bessel filter) before digitized and stored on a hard disk of a Digidata 1200A acquisition board (Axon Instruments)-equipped IBM pentium computer for subsequent analysis. All experimental parameters, such as pulse generation and data acquisition, were controlled using pClamp 6.04 software (Axon Instruments).

Solutions and drugs

The external bath solution contained (in mM): 140 NaCl, 5 KCl, 1.3 $CaCl_2$, 1 $MgCl_2$, 20 HEPES, and 10 glucose, and was adjusted to pH 7.3 with NaOH. This bath solution was used as the internal pipette solution for inside-out recordings. The internal pipette solution contained (in mM): 140 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 EGTA and was adjusted to pH 7.3 with KOH. This pipette solution was used as the bath solution for inside-out recordings. For fast drug application, CsA was rapidly applied with a superfusion system using a piezoelectric-driven micromanipulator (P-287.70, Physik Instrumente, Waldbronn, Germany) as described previously (Choi et al, 2003). CsA was obtained from Novartis (Stein, Switzerland). Calcineurin autoinhibitory peptide (Calbiochem, San Diego, CA, USA) was dissolved in distilled water and directly added to the internal pipette solution. Cypermethrin (Calbiochem) was dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions. The concentration of DMSO in the final dilution was less than 0.1%, and this DMSO concentration had no effect on Kv1.5 and Kv1.3 currents.

Data analysis

For analysis, Origin 6.1 software (Microcal Software, Inc., Northampton, MA, USA) was used. Activation curves were fitted with a Boltzmann equation:

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

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

Interaction kinetics between drug and channel was described on the basis of a first-order blocking scheme as previously described (Snyders & Yeola, 1995). From this concept, the apparent IC_{50} and Hill coefficient n were obtained by fitting concentration dependence data to the following equation:

$$f=1/[1+([D]/IC_{50})^n] \quad (2)$$

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

$$1/\tau_D=k_{+1}[D]+k_{-1} \quad (3a)$$

$$K_d=k_{-1}/k_{+1} \quad (3b)$$

in which τ_D is the drug-induced time constant.

Data are expressed as mean s.e. Student's t-test and analysis of variance were used for statistical analysis. Statistical significance was considered at $p<0.05$.

RESULTS

Concentration-dependent block of Kv1.5 by CsA

Fig. 1A shows the superimposed-original Kv1.5 current traces obtained by 250-ms depolarizing pulses to +50 mV under control conditions and in the presence of CsA. In the absence of the drug, Kv1.5 currents were rapidly activated, reached a maximum peak, and then slowly inactivated, while the depolarizing pulse was maintained as described previously (Choi et al, 2000). Thus, the amplitude of the current measured at the end of the 250-ms depolarizing pulse to +50 mV decreased by 91.9±0.11% (n=15). In the presence of the drug (0.3~10 μM), the peak current ampli-

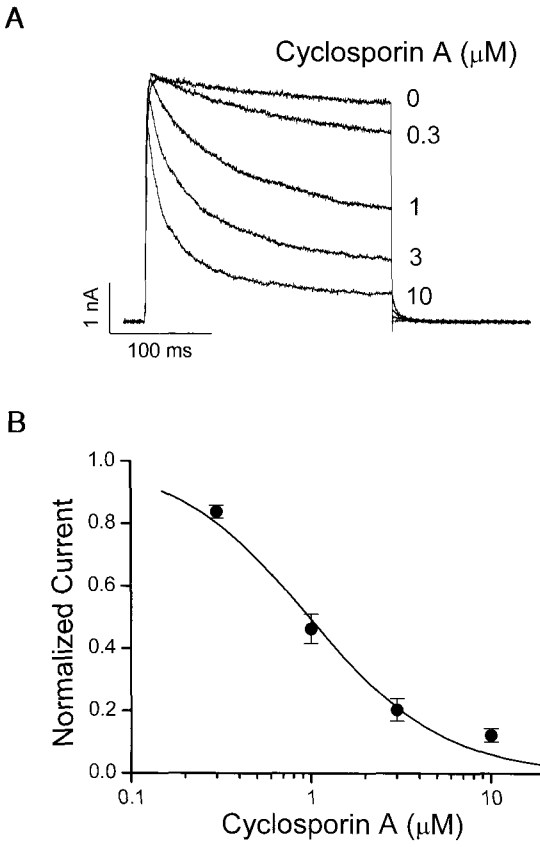

Fig. 1. Concentration-dependent block of Kv1.5 whole-cell currents expressed in CHO cells by CsA. (A) Superimposed currents were elicited by applying 250 ms depolarizing pulses from a holding potential of −80 mV to +50 mV every 10 s in the absence and presence of 0.3, 1, 3, and 10 μM CsA. (B) Concentration-response curve for the block of Kv1.5 current by CsA. The drug-induced block was measured at the end of a 250 ms depolarizing pulse of +50 mV and normalized by current under control conditions. The normalized currents were fitted with the Hill equation which yielded an IC_{50} of 1.0±0.1 μM and a Hill coefficient of 1.2±0.2 (n=7) for CsA. Data are expressed as mean±s.e.

tude was not affected by CsA at the concentrations used. However, the current decay was much faster than that observed without the drug, and steady-state currents measured at the end of the 250-ms depolarizing pulse decreased in a concentration-dependent manner. Therefore, the current measured at the end of 250-ms depolarizations was used to construct concentration-response curves for channel block. A nonlinear least-squares fit of the Hill equation to the concentration-response data yielded an apparent IC_{50} value of 1.0±0.1 μM and a Hill coefficient of 1.2±0.2 (n=7) for CsA at +50 mV (Fig. 1B).

To assess the reversibility of the drug effect, the single pulse was repeated while CsA, separated by washout periods, was applied. As shown in Fig. 2, when switched to solutions containing the drug, steady state was reached within 3 min. The washout of CsA by perfusion of the drug-free solution was obtained within 4 min. The currents recovered from the steady-state block by CsA to 88.1±0.1% (n=5) of the control. Therefore, the block was reversible on washout with little rundown of the current observed under

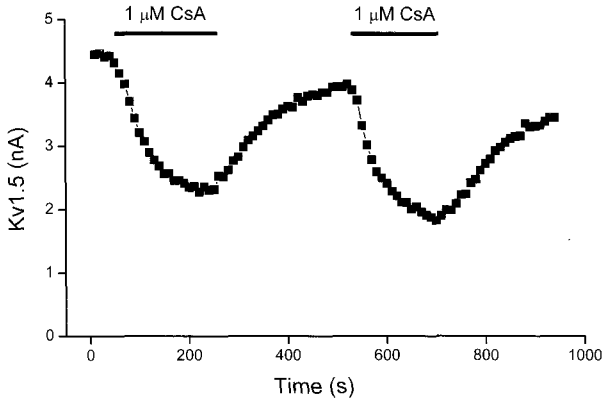

Fig. 2. Reversible block of Kv1.5 by CsA. Time course of block in the presence of CsA. The complete recovery from block was observed after washout of the drug. The steady-state amplitudes of the current were plotted as a function of time. The bar indicates the time of CsA application.

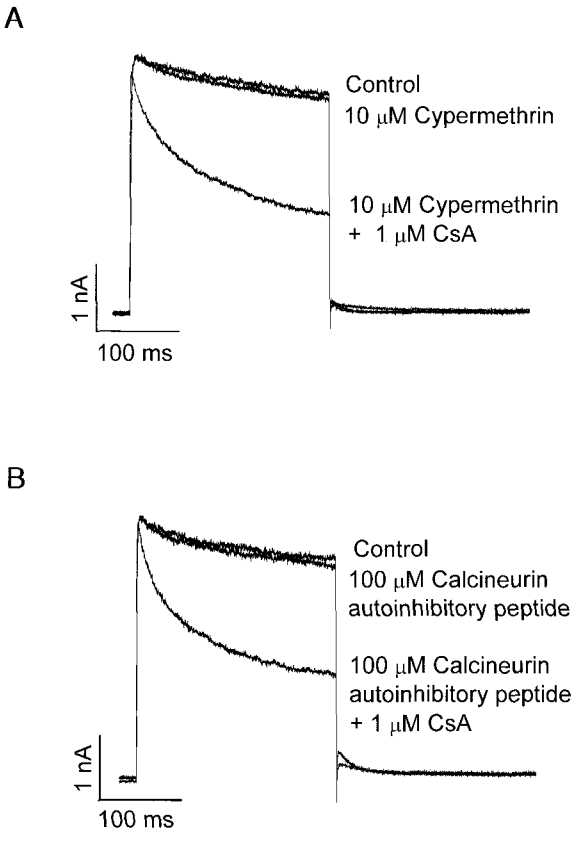

Fig. 3. The effects of calcineurin inhibitors, cypermethrin and calcineurin autoinhibitory peptide on the block of Kv1.5 currents by CsA. Whole-cell currents were elicited by applying 250 ms depolarizing pulses from a holding potential of −80 mV to +50 mV every 10 s. (A) Control current, current recorded after 5 min of exposure to 10 μM cypermethrin, and current measured by treatment of 1 μM CsA are shown. (B) Control current recorded immediately after membrane rupture, current recorded 5 min after membrane rupture, and current measured by treatment of 1 μM CsA are shown. Calcineurin autoinhibitory peptide (100 μM) was included in pipette solution.

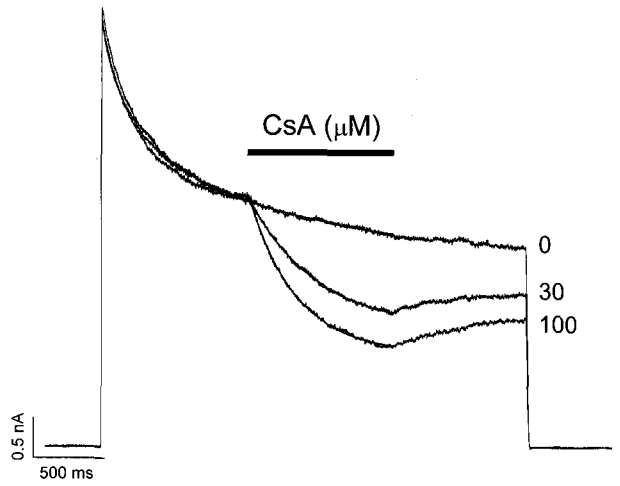

Fig. 4. The time course of Kv1.5 current block by fast application of CsA. The whole-cell currents were elicited by applying 3,000 ms depolarizing pulses from a holding potential of −80 mV to +50 mV every 10 s. After a delay of about 1,000 ms, CsA was rapidly applied during the depolarizing pulse. The bar indicates the time of application of CsA.

these conditions.

The effects of calcineurin inhibitors on the block of Kv1.5 by CsA

The Kv1.5 channel could be phosphorylated and dephosphorylated, and CsA has been classified as a phosphatase inhibitor. To elucidate whether calcineurin was involved in CsA-induced block of Kv1.5, we investigated further the effects of calcineurin inhibitors, such as cypermethrin, in bath solution and calcineurin autoinhibitory peptide in pipette solution. Fig. 3A shows the effects of cypermethrin on the block of Kv1.5 by CsA. A 10-min exposure to 10 μM cypermethrin did not induce the block of Kv1.5. After a 10-min exposure to 10 μM cypermethrin, 1 μM CsA blocked the steady-state current of Kv1.5 measured at the end of the depolarizing pulse of +50 mV by 54.7% (n=5), which was not significantly different from the block induced by CsA (53.7%) in the absence of cypermethrin (see Fig. 1). Fig. 2B shows the effects of 100 μM calcineurin autoinhibitory peptide in the pipette solution. Ten min after membrane rupture to allow for complete dialysis, the steady-state amplitude of Kv1.5 was not affected, compared with the control measured immediately after membrane rupture. By adding 1 μM CsA to the bath solution, the steady-state amplitude of Kv1.5 was decreased by 52.7%. The lack of effects of cypermethrin and calcineurin autoinhibitory pep-

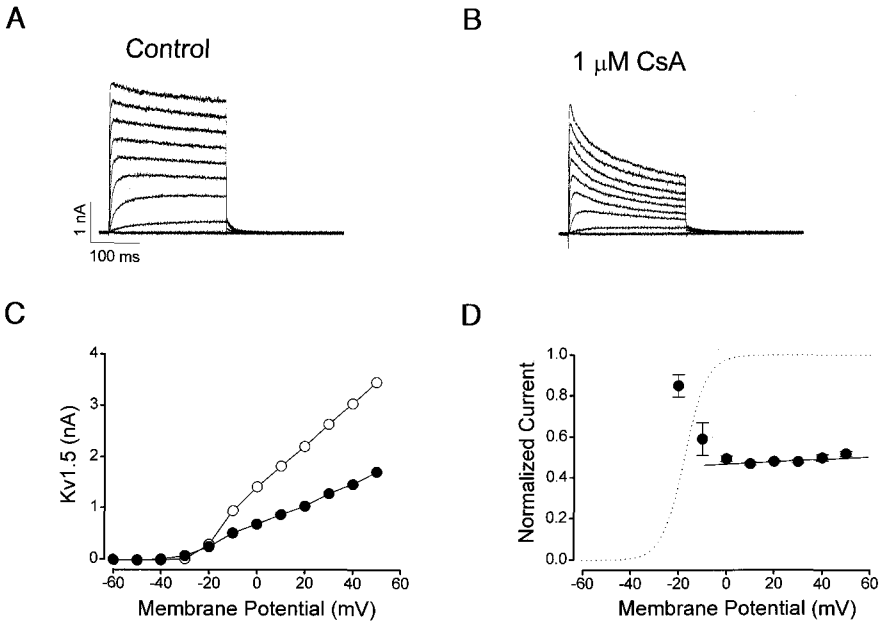

Fig. 5. Voltage dependence of the Kv1.5 block by CsA. The whole-cell currents were elicited by applying 250 ms depolarizing pulses between −60 mV to +50 mV in 10 mV increments every 10 s from a holding potential of −80 mV under control conditions (A) and after the addition of 1 μM CsA (B). (C) Resultant I-V relationships were taken at the end of the test pulses (○, Control; ● 1 μM CsA). (D) Normalized block shown as relative current ($I_{CsA}/I_{control}$) from data in C. For potentials positive to 0 mV, the voltage dependence was linear fitted and yielded the slope value equal to zero (n=6). The dotted line represents the activation curve of typical Kv1.5 under control conditions, which was obtained from a deactivating tail current amplitude at −40 mV after 250 ms depolarizing pulses to potentials between −60 to +50 mV in steps of 10 mV from a holding potentials of −80 mV were applied. Data show the conductance at each step voltage standardized to the conductance at +50 mV. Data are expressed as mean±s.e.

tide on CsA-induced block of Kv1.5 strongly indicates that CsA directly blocks Kv1.5 in a calcineurin-independent manner.

Effects of fast application of CsA on Kv1.5

Fast drug application system was used to confirm that Kv1.5 block by CsA occurred in a calcineurin-independent manner. As shown in Fig. 4, the cells were held at −80 mV and depolarized to +50 mV for 3,000 ms every 10 s. After a delay of 1000 ms, fast application of CsA during the depolarizing pulse led to a rapid and reversible block of Kv1.5. The onset time constants of the CsA-induced block were concentration-dependent with time constants of 463.8± 9.1 ms and 325.4±10.3 ms for 30 μM and 100 μM CsA (n= 4), respectively. This system revealed onset of block on a millisecond time scale. Although the currents did not recover to the control amplitude at the end of the depolarization pulse, however, the complete recovery of the currents after washout period of 10 s was observed between successive current sweeps as shown in Fig. 4.

Voltage-dependent block of Kv1.5 by CsA

Fig. 5 shows the effect of CsA on current-voltage (I~V) relationship. Under control conditions, the I~V relationship was sigmoidal for depolarizing pulses between 30 and 0 mV and almost linear for depolarizing pulses greater than 0 mV (Fig. 5A and C). In the presence of 1 μM CsA, the block of steady-state currents was observed in the entire voltage range over which Kv1.5 was activated, as shown in Fig. 5B and C. By plotting the relative current ($I_{CsA}/I_{control}$) versus potential (Fig. 4D), a high degree of block with a strong voltage dependence was observed between −30 and 0 mV, which corresponds to the voltage range of the opening of channels. This suggests that CsA-induced block of Kv1.5 currents occurs preferentially after channels open. However, the block of Kv1.5 channels by CsA in the range of voltages between 0 and +50 mV, where channels are fully activated, did not show voltage dependence. The linear curve fitting of the data at potentials greater than 0 mV (Fig. 5D, solid line) yielded a value approximately equal to zero for the slope of the line: 49.5±.4 % of the control value at 0 mV and 51.8±1.2 % of the control at +50 mV (n=6, ANOVA, $p>0.05$).

Time dependence of CsA-induced block of Kv1.5

The block of the Kv1.5 currents was not prominent at the beginning, however, but developed gradually during the voltage steps, indicating that the block by CsA is time-dependent. Fig. 6A shows superimposed current traces under control conditions and in the presence of various concentrations of CsA. To determine the time course of development of the block of Kv1.5 current with different concentrations of CsA, the current traces in Fig. 6A were modified by the expression ($I_{control}$-I_{drug}) as shown in Fig. 6B. The resultant data clearly showed time-dependent increase in block during the depolarization which are more evident with higher concentration of CsA, and were well fitted to a single exponential function which yielded a time constant for Kv1.5 current block (τ_D). A plot of the reciprocal of τ_D at +50 mV versus each concentration yielded an apparent association rate constant (k_{+1}) of 7.0±1.5 $M^{-1}s^{-1}$, and an apparent dissociation rate constant (k_{-1}) of 8.1±0.5 s^{-1}

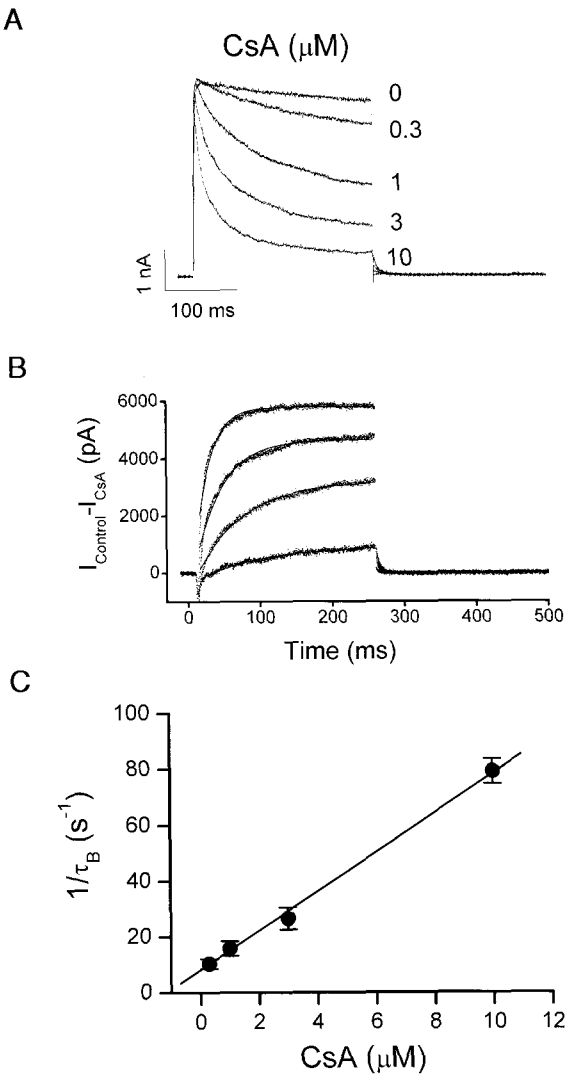

Fig. 6. Time constant of block as a function of drug concentrations. (A) Superimposed currents were elicited by applying 250 ms depolarizing pulses from a holding potential of −80 mV to +50 mV every 10 s in the absence and presence of 0.3, 1, 3, and 10 μM CsA. (B) The drug-induced time constants (τ_D) were obtained from a single exponential fitting after a correction of I_{drugs}-$I_{control}$. (C) The reciprocals of drug-induced time constants were plotted versus CsA concentrations. The solid line represents the least-squares fit of the data to the relation $1/\tau_D=k_{+1}[D]+k_{-1}$. An apparent association rate constant (k_{+1}) of 7.0±1.5 $\mu M^{-1}s^{-1}$ and dissociation rate constant (k_{-1}) of 8.1±0.5 s^{-1} (n=5) were obtained from the slope and intercept values of the fitted line. The theoretical K_d value derived by k_{-1}/k_{+1} yielded 1.2 μM. Data are expressed as mean±s.e.

(Fig. 6C). On the basis of the first order interaction between CsA and Kv1.5 (see *Methods*), the theoretical K_d value derived by k_{-1}/k_{+1} yielded 1.2 μM. Although the derived K_d of 1.2 μM was independent of the apparent IC_{50} of 1.0 μM obtained from the concentration-response curve shown in Fig. 1B, the two values are reasonably close.

Fig. 7 shows the superposition of the tail currents recorded at a 250-ms repolarizing potential of 40 mV after

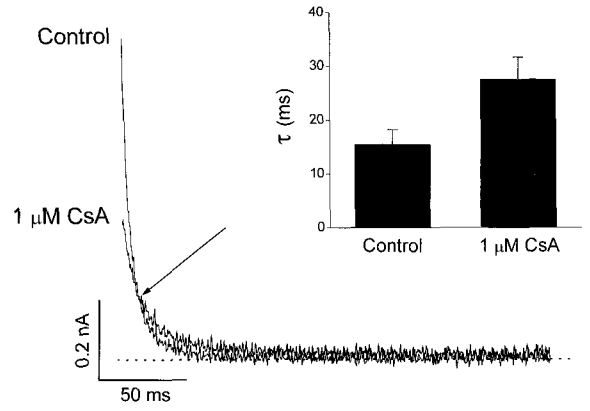

Fig. 7. Effects of CsA on the deactivation time constant of Kv1.5. Tail currents were recorded after a 250 ms repolarizing pulse of −40 mV after a 250 ms depolarizing pulse of +50 mV from a holding potential of −80 mV in the absence and presence of 1 μM CsA. By superimposing the two tail currents in the absence and presence of CsA, tail crossover phenomenon (indicated by the arrow) was observed. The values of deactivation time constants were obtained from a single exponential fit. Under control conditions and in the presence of 1 μM CsA, the tail current declined with a time constant of 15.5±2.7 ms (n=5) and 27.5±4.2 ms (n=5), respectively. Those two values were significantly different ($p<0.05$). The dotted line represents zero level of current. Data are expressed as mean±s.e.

a 250 ms depolarizing pulse of +50 mV from a holding potential of 80 mV under control conditions and in the presence of 1 μM CsA. Under control conditions, the tail current declined quickly with a time constant of 15.5±2.7 ms (n=5), and nearly completely deactivated during the 250 ms repolarizing potential of 40 mV. In the presence of CsA (1 μM), the initial peak amplitude of tail current was reduced. The subsequent decline of the current was slower (27.5 4.2 ms, n=5, Student's t-test, $p<0.05$) than in control conditions, resulting in the tail crossover phenomenon.

Use dependence of CsA action on Kv1.5

Fig. 8 shows the use-dependent block of Kv1.5 by CsA. Fifteen repetitive 125-ms depolarizing pulses of +50 mV from a holding potential of 80 mV were applied at two different frequencies, 1 and 2 Hz. Under control conditions, the peak amplitude of the Kv1.5 current decreased by 6.3 0.6 (n=6) at a frequency of 1 Hz and by 12.0±0.9 (n=6) at a frequency of 2 Hz in a weak frequency-dependent manner. In the presence of 1 M CsA, the peak amplitude of Kv1.5 progressively decreased by 13.0±0.9 (n=6) and 27.7± 1.6 (n=6) at 1 and 2 Hz, respectively in a strong frequency-dependent manner. However, the peak current amplitude at the first pulse was not significantly affected, indicating that there was no tonic block by CsA.

Concentration-dependent block of Kv1.3 by CsA

Fig. 9 shows superimposed Kv1.3 current traces produced by a 250-ms depolarizing pulse to +50 mV under control conditions and in the presence of various concentrations of CsA. CsA induced a reduction in the steady-state current amplitude during the depolarizing pulse with little effect

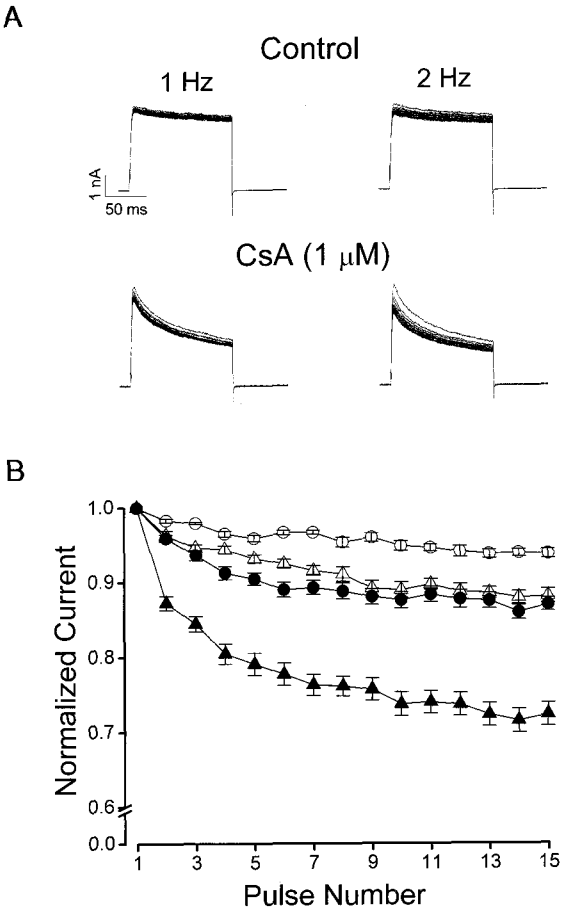

Fig. 8. Use-dependent block of Kv1.5 current by CsA. (A) Fifteen repetitive 125 ms depolarizing pulses of +50 mV from a holding potential of −80 mV were applied at two different frequencies, 1 and 2 Hz under control conditions (○, △) and in the presence of 1−M CsA (●, ▲). (B)The peak amplitudes of current at every pulse were normalized by the peak amplitudes of current obtained at the first number of pulse and then plotted versus the pulse numbers. Data are expressed as mean±s.e. (n=6).

on the peak amplitude. The current amplitude measured at the end of a 250-ms depolarizing pulse was used as an index of block. A nonlinear least-squares fit of the Hill equation (equation 1) to the individual data points yielded an IC_{50} value of 1.1±0.06 μM and a Hill coefficient of 1.4± 0.06 (Fig. 9B, n=6).

Fig. 10 shows the effects of CsA on Kv1.3 currents recorded from inside-out patches. In the presence of 1 μM CsA, the block of Kv1.3 currents was also characterized by an acceleration in the apparent rate of current decay with little effect on peak amplitude, as shown in whole-cell recordings (Fig. 9). CsA (1 μM) blocked the steady-state current of Kv1.3 at the end of a depolarizing pulse of +50 mV by 77.5±4.9% of the control value (n=4), suggesting that the drug acts either on an intracellular site or can pass through the membrane to its site of action somewhere within the membrane.

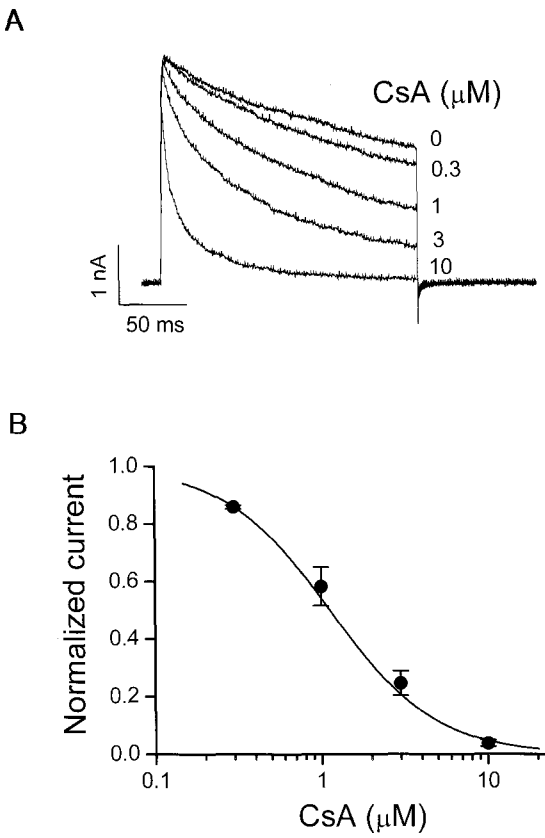

Fig. 9. Concentration-dependent block of Kv1.3 whole-cell currents expressed in CHO cells by CsA. (A) Superimposed currents were elicited by applying 250 ms depolarizing pulses from a holding potential of −80 mV to +50 mV every 30 s in the absence and presence of 1, 3, 10, and 30 μM FK506. (B) Concentration-response curve for the block of Kv1.3 current by CsA. The drug-induced block was measured at the end of a 250 ms depolarizing pulse of +50 mV and normalized by current under control conditions. The normalized currents were fitted with the Hill equation which yielded an IC_{50} of 1.1±0.06 μM and a Hill coefficient of 1.4±0.06 (n=6) for CsA. Data are expressed as mean±s.e.

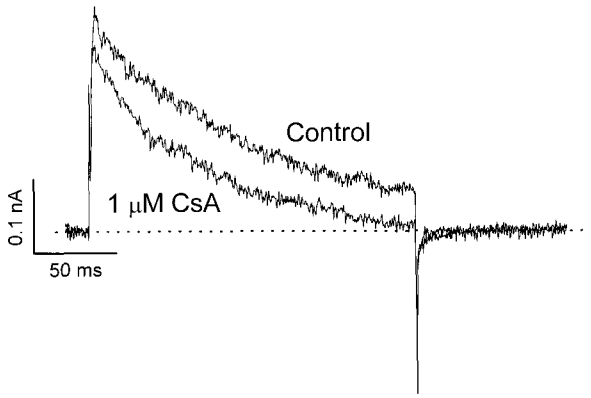

Fig. 10. Effects of CsA on Kv1.3 currents recorded from inside-out patches. Superimposed original currents of Kv1.5 were elicited by applying 250 ms pulses from a holding potential of −80 mV to +50 mV every 30 s in the absence and presence of 1 μM CsA. CsA (1 μM) blocked the steady-state current of Kv1.3 at the end of a depolarizing pulse of +50 mV by 22.5±4.9% of the control value (n=4).

DISCUSSION

Primary purpose of this study was to determine the direct effect of CsA on cloned K^+ channel, Kv1.5 and Kv1.3, and results demonstrated that CsA produced a concentration-, time-dependent, and reversible block of Kv1.5 and Kv1.3 channels. These results suggest that CsA blocks cloned K^+ channels Kv1.5 by mechanism not involving suppression of serine/threonine protein phosphatase calcineurin activity, but by distinct mechanism involving direct open channel block.

CsA is a potent immunosuppressant widely used for preventing rejections after organ transplantation and for the treatment of various autoimmune diseases (Sigal & Dumont, 1992; Matsuda & Koyasu, 2000). The mechanism of immunosuppressive action of this drug has been thought to be mediated by calcineurin-dependent mechanism. In the present study, however, the following results indicated that CsA directly blocked Kv1.5 currents in a phosphorylation-independent manner. First, in our experiments, none of the calcineurin inhibitors tested prevented the effects of CsA on the Kv1.5 channel activity. The Kv1.5 channel has multiple phosphorylation site for PKC, PKA and tyrosine kinase, and is regulated via serine/threonine and tyrosine phosphorylation (Swanson et al, 1990; Tseng-Crank et al, 1990). It has been reported that CsA increases the phosphorylation of endogenous proteins in brain tissue by inhibiting phosphatase activity (Steiner et al, 1992). In addition, CsA can activate protein kinase C (Demeule et al, 1994; Haller et al, 1994), however, phosphorylation by PKA activatior has no effect on the amplitude of Kv1.5 currents (Mason et al, 2002) and activation of PKC by PMA has minimal effect, when Kv1.5 was expressed alone in *Xenopus* oocyte (Williams et al, 2002). These results are inconsistent with our data, suggesting that CsA blocked Kv1.5 independently of phosphorylation and had a direct blocking effect on the Kv1.5 channel. Second, our experiments were performed in whole-cell recordings in which Ca^{2+} was buffered by EGTA to resting concentration (<10 nM). Because the catalytic activity of calcineurin requires a significant increase of intracellular free Ca^{2+} concentration, our experimental conditions suggest calcineurin-independent block of Kv1.5 by CsA. Finally, the rapid onset and reversibility of the block of Kv1.5 in whole-cell recordings indirectly suggest that block was not phosphorylation-dependent. Furthermore, in excised inside-out patch which is devoid of diffusible cytosolic molecules, the rapid and reversible effect of CsA is also consistent with a direct interaction with Kv1.5 channel rather than through a phosphorylation-dependent mechanism. To obtain more direct evidence, fast drug perfusion system was applied to estimate the time course of current block. Upon fast application of CsA, the onset of block occurred on a millisecond time scale. Because modulation of ion channels by phosphorylation takes seconds to minutes (Chung & Schlichter, 1997; Choi et al, 2002; Williams et al, 2002), it is again likely that the rapid current reduction was not due to involvement of second messenger pathways such as, phosphorylation and dephosphorylation, but rather a direct action on Kv1.5 channels.

In principle, channel block can be explained by two basic mechanisms. One mechanism proposes that the drug enters

and binds in the pore as an open channel blocker, and the other suggests that the action of the drug takes place at an allosteric site on the intra- or extracellular surface. In this study, the following characteristics of the CsA-induced block of Kv1.5 suggest that CsA preferentially interacts with the open state of Kv1.5; First, CsA accelerated the rate of Kv1.5 current decay during a depolarizing pulse, suggesting an interaction with the open state of the channel. Second, CsA had no effect on the peak amplitude of the current at the onset of a depolarizing pulse, indicating that CsA did not bind to the closed or resting state of Kv1.5. Third, the block induced by CsA was voltage-dependent and increased steeply in the voltage range of channel activation. Open channel blocking mechanisms are associated with the charged form of drug molecules and a voltage-dependence of the block (Snyders & Yeola, 1995). If the non-charged form of a drug accesses its binding site on Kv1.5 from the intracellular surface, an additional voltage dependence of block should not be observed in the voltage range where the Kv1.5 channel is fully activated. However, a high degree of block with a strong voltage dependence was still detected in the voltage range of channel opening (Choi et al, 2002). This explanation could be applied to CsA, because CsA is predominately in an uncharged neutral form at physiological pH (Sigal & Dumont, 1992), resulting in independence of the transmembrane electric field. As expected, the block of Kv1.5 channels by CsA in the range of voltages, where channels are fully activated, did not show voltage dependence. Fourth, CsA slowed the deactivation time course, resulting in a tail crossover phenomenon. This phenomenon suggests an interaction between CsA and the open state of Kv1.5 (Delpon et al, 1997; Valenzuela et al, 1997; Choi et al, 2000). Fifth, the blocking action of CsA was use-dependent, with effects enhanced at higher rates of channel activation. This is also consistent with the actions of CsA on the open state of Kv1.5 (Delpon et al, 1997; Choi et al, 2002).

The therapeutic plasma concentration of CsA in patients with transplantation is in the range of $0.1 \sim 0.2\,\mu$M. We demonstrated a concentration-dependent decrease in Kv1.5 currents with an IC_{50} of $1.0\,\mu$M, and this effect appears to be of no clinical relevance. Nonetheless, our results indicate small but significant reduction of Kv1.5 current at low concentration of $0.3\,\mu$M. Moreover, CsA can accumulate in higher concentrations in mononuclear cells, including T-lymphocytes and in heart tissue than in plasma (Niederberger et al., 1983). With the above considerations, therefore, our results would help understand molecular mechanisms responsible for immunosuppressive effects of CsA.

In conclusion, we report for the first time a novel calcineurin-independent pharmacological property of CsA, indicating the direct blocking effects of CsA on cloned Kv1.5 channels.

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