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SHORT COMMUNICATION

Voltage-dependent biphasic effects of chloroquine on delayed rectifier K^+-channel currents in murine thymocytes

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Abstract Lymphocytes are of rich in delayed rectifier K^+-channels (Kv1.3) in their plasma membranes, and the channels play crucial roles in the lymphocyte activation and proliferation. Since chloroquine, a widely used anti-malarial drug, exerts immunosuppressive effects, it will affect the channel currents in lymphocytes. In the present study, employing the standard patch-clamp whole-cell recording technique, we examined the effects of chloroquine on the channels expressed in murine thymocytes. Published papers report that chloroquine will inhibit voltage-dependent K^+-channel currents by plugging into the open-pore. We observed, indeed, that chloroquine suppressed the pulse-end currents of Kv1.3-channels at higher voltage steps. Surprisingly, however, we found that the drug enhanced the peak currents at both higher and lower voltage steps. Since chloroquine showed such biphasic effects on the thymocyte K^+-channels, and since those effects were voltage dependent, we examined the effects of chloroquine on the activation and the inactivation of the channel currents. We noted that chloroquine shifted both the activation and the inactivation curves toward the hyperpolarizing potential, and that those shifts were more emphasized at lower voltage steps. We conclude that chloroquine facilitates both the activation and the inactivation of Kv1.3-channel currents in thymocytes, and that those effects are voltage dependent.

Keywords Chloroquine · Lymphocytes · Kv1.3-channel · Activation · Inactivation

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Introduction

Despite the emergence of the drug-resistant parasitic strains, chloroquine remains the most widely used anti-malarial drug in the world [1]. Because of its immunosuppressive properties, chloroquine is also used to treat autoimmune disorders, such as rheumatoid arthritis [2] and systemic lupus erythematosus [3]. In those diseases, chloroquine suppresses the activity of lymphocytes by reducing their proinflammatory cytokine production [4], or it depresses the mitogen-driven proliferation of lymphocytes [5]. Patch-clamp studies revealed that lymphocytes predominantly express delayed rectifier K^+-channels (Kv1.3) in their plasma membranes [6]. The channels generate the K^+-diffusion potential across the plasma membranes, and thus play roles in regulating the resting membrane potential and controlling the cell volume. Using the selective channel inhibitors, later studies have further demonstrated that the channels also play crucial roles in triggering the calcium influx necessary for the lymphocyte activation and proliferation [7, 8]. Since chloroquine exerts immunosuppressive effects in lymphocytes, we assume here that this drug would affect the channel currents in those cells. To test this, employing the standard patch-clamp whole-cell recording technique, we examined the effects of chloroquine on the channels expressed in murine thymocytes.

During its cardiotoxic complications, such as prolonged QT interval syndrome, chloroquine inhibits both voltage-dependent [9, 10] and inward rectifier [11] K^+-channel currents in ventricular myocytes. As a molecular mechanism of the inhibition, published papers have proposed that chloroquine plugs into the open-pore of the channel and, thus, facilitates the inactivation of the channel currents [10, 11]. However, we know little about chloroquine's effect on the activation that would greatly influence the magnitude of

the peak currents. Unveiling the crystal structures of voltage-dependent K^+-channels [12], recent studies have demonstrated that the activation and the inactivation gates are closely located to each other within a pore-forming domain of the channel [13, 14]. The studies further demonstrated that those gates are functionally coupled, indicating chloroquine that facilitates the inactivation will also affect the activation. To address this issue, we have applied the appropriate voltage protocols to the thymocytes to examine the effects of chloroquine on the activation and the inactivation of Kv1.3-channel currents. Here, we demonstrate for the first time that chloroquine facilitates both the activation and the inactivation of the channel currents. We also show that those effects of chloroquine are voltage-dependent.

Methods

Cell sources and preparation

Male *ddy* mice (4–5 weeks old), supplied by Japan SLC (Shizuoka, Japan) were deeply anaesthetized with isoflurane and then sacrificed by cervical dislocation. The protocol for animal use was approved by the Animal Care and Use Committee of Tohoku University Graduate School of Medicine. Single thymocytes were isolated from murine thymus as described previously [15]. Briefly, thymic lobes were harvested from the mice, gently teased apart with forceps in standard external (bathing) solution containing (in mM): NaCl, 145; KCl, 4.0; $CaCl_2$, 1.0; $MgCl_2$, 2.0; Hepes, 5.0; bovine serum albumin, 0.01% (pH 7.2 adjusted with NaOH), and disseminated by repetitive pipetting. After removing large tissue pieces, cells were washed twice by gentle centrifugation for 1 min and resuspended in the standard external solution. They were maintained at room temperature (22–24°C) for use within 4 h.

Electrical setup and patch-clamp recordings

We conducted standard whole-cell patch-clamp recordings using an EPC-9 patch clamp amplifier system (HEKA Electronics, Lambrecht, Germany), as described previously [16, 17]. Using a two-stage puller (PP-83 puller; Narishige, Tokyo, Japan), we pulled patch pipettes from plain haematocrit glass capillaries. The patch pipette resistance was 4–6 MΩ when filled with the internal (patch pipette) solution containing (in millimolar): KCl, 145; $MgCl_2$, 1.0; EGTA, 10; Hepes, 5.0 (pH 7.2 adjusted with KOH). After a giga-seal formation, we applied suction briefly to the pipette to rupture the patch membrane. The series resistance of whole-cell recordings was maintained below 10 MΩ during experiments. Peak currents were normalized by the membrane capacitance and expressed as the current densities (pA/pF). Pulse-end currents were expressed as percentages of the peak currents. All experiments were carried out at room temperature.

Drug delivery

We purchased margatoxin from Peptide Institute (Osaka, Japan), chloroquine diphosphate (chloroquine) from Wako Pure Chem Ind. (Osaka, Japan). They were separately dissolved in the external solution at the final concentrations of 100 nM and 10 μM, respectively. We delivered one of the reagents to the cells by the standing hydrostatic pressure of 3 cmH_2O from a nearby pipette, as described previously [17]. Briefly, using a three-dimensional hydraulic manipulator (M-103; Narishige), the application pipette initially positioned outside the bathing solution was brought to within 10 μm of the cell surface. Then, to wash out the reagents, the pipette was brought up outside the bathing solution. Whole-cell membrane currents were recorded before and after 1 min exposure to these reagents and after a 2 min washout. To rule out the possibility that the observed effect just resulted from the procedure of reagent application, we simply applied the external solution to the cells and confirmed the absence of any significant changes in the channel currents (Fig. 1a).

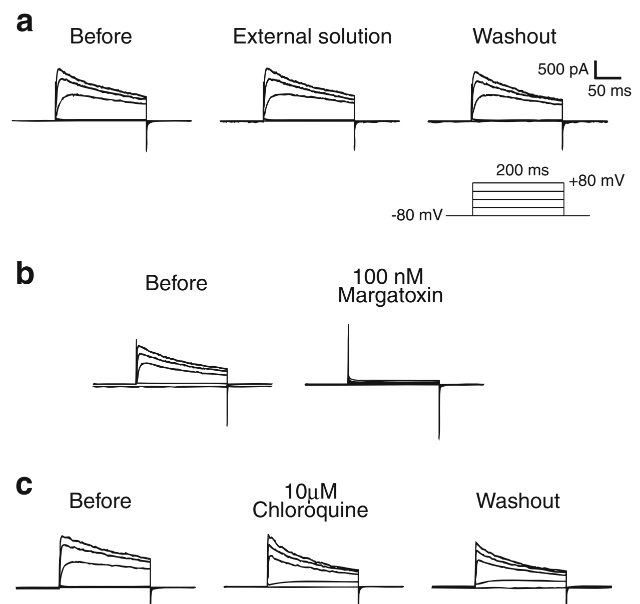

Fig. 1 Effects of margatoxin and chloroquine on voltage-dependent outward currents in murine thymocytes. Effects of the external solution containing no reagents (**a**), 100 nM margatoxin (**b**) and 10 μM chloroquine (**c**). Typical whole-cell current traces recorded before and after the reagent application, and/or after its washout. The currents were elicited from the holding potential of −80 mV to the various voltage steps as depicted in the voltage protocol. Each pulse was applied for a 200-ms duration between 10-s intervals

Statistic analyses

Data were analyzed using PulseFit software (HEKA Electronics, Lambrecht, Germany), IGOR Pro (WaveMetrics, Lake Oswego, OR, USA) and Microsoft Excel (Microsoft, Redmond, WA, USA) and reported as mean ± SEM. Statistical significance was assessed by two-way ANOVA followed by Dunnett's or Student's *t* test. A value of $P < 0.05$ was considered significant.

Results

Identification of Kv1.3-channel currents in thymocytes and the suppressive effect of chloroquine

We started our protocols by applying 200-ms constant step-voltage pulses to single thymocytes (Fig. 1). Predominant ion channels in thymocytes are voltgage-dependent K^+-selective channels (Kv) [6]. Stepwise changes in the membrane potential, from the holding potential of −80 mV to the various depolarizing potential levels, evoked membrane currents showing voltgage-dependent activation and inactivation characteristic to this type (Fig. 1a). Since the application of margatoxin, a relatively selective Kv1.3-channel inhibitor, almost totally abolished the currents (Fig. 1b), we identified them with the Kv1.3-channel currents.

Therapeutic doses of chloroquine typically result in plasma concentrations between 2 and 3 μM, with peak levels up to 5 μM [18]. However, according to several in vitro studies, single cells require at least 10 μM chloroquine to effectively elicit its inhibitory properties on K^+-channel currents [9–11]. Thus, we applied 10 μM chloroquine to the thymocytes to determine its effects on Kv1.3-channel currents (Fig. 1c). Similar to the effects demonstrated by the other quinoline derivatives, such as quinidine [19], chloroquine suppressed the pulse-end currents (Fig. 1c). The current suppression did not recover after the drug withdrawal, indicating that the chloroquine's effect is essentially irreversible (Fig. 1c).

Effects of chloroquine on thymocyte Kv1.3-channel currents at higher and lower voltgage steps

To focus on chloroquine's effect on the pulse-end current decrease, we applied single pulses longer than 200 ms. Pulses longer than 1 s required too much time for the recovery from the inactivation (data not shown); thus, we applied 500-ms pulses at the longest (Fig. 2). Since chloroquine tended to suppress the pulse-end currents more obviously at higher voltgage steps (Fig. 1c), we examined the effects at the higher voltgage step of +40 mV (Fig. 2A). As expected from the result in Fig. 1c, chloroquine markedly suppressed the pulse-end currents. The ratio of pulse-end currents per peak currents was significantly decreased from 40.2 ± 4.9 to 16.8 ± 2.8% ($n = 6$, $P < 0.05$; Fig. 2Ab). Surprisingly, however, chloroquine enhanced the peak currents with significant increase in the current densities (from 155 ± 19 to 237 ± 19 pF/pA, $n = 6$, $P < 0.05$; Fig. 2Ac).

In contrast to the pulse-end currents, chloroquine tended to enhance the peak currents at lower voltgage steps (Fig. 1c). To focus on such chloroquine's effect on the peak currents, we then examined the effects at the lower voltgage step of −40 mV (Fig. 2B). At this voltgage step, chloroquine failed to suppress the pulse-end currents (Fig. 2Bb). However, it markedly enhanced the peak currents with more than twofold increase in the current densities (from 6.1 ± 1.5 to 17.6 ± 1.9 pF/pA, $n = 6$, $P < 0.05$; Fig. 2Bc), followed by a further increase after the drug withdrawal. These results indicate that chloroquine shows biphasic effects on thymocyte Kv1.3-channel currents in a voltgage-dependent manner; suppresses the pulse-end currents at higher voltgage steps, but enhances the peak currents at both higher and lower voltgage steps.

At the voltgage step of −70 mV that is around the resting membrane potential of thymocytes [20], the channel currents were not elicited (Fig. 2C) and chloroquine did not affect it.

Effects of chloroquine on thymocyte Kv1.3-channel currents in short pulse protocols

To rule out the influence of the inactivation factors induced by the long preceding pulses, we also applied pulses much shorter than 200 ms (Fig. 3). Since 10-ms pulses were too short to induce the inactivation factors, but were long enough to obtain the peak currents (Fig. 3Aa, Ba), we applied this duration of pulses. At the higher voltgage step of +40 mV, chloroquine did not alter the peak currents (Fig. 3Ab). However, at the lower voltgage step of −20 mV, it markedly enhanced the peak currents with significant increase in the current densities (from 76 ± 9.2 to 155 ± 24 pF/pA, $n = 6$, $P < 0.05$; Fig. 3Bb). At the resting membrane potential of −70 mV, the channel currents were not elicited (Fig. 3C) and chloroquine did not affect it.

Effects of chloroquine on activation and inactivation of thymocyte Kv1.3-channel currents

Since chloroquine biphasically affects both the peak and the pulse-end currents of thymocyte Kv1.3, the drug was expected to modify both the activation and the inactivation of the channel currents. Therefore, employing the appropriate voltage protocols, we drew activation and inactivation curves of the channel currents, and then examined the

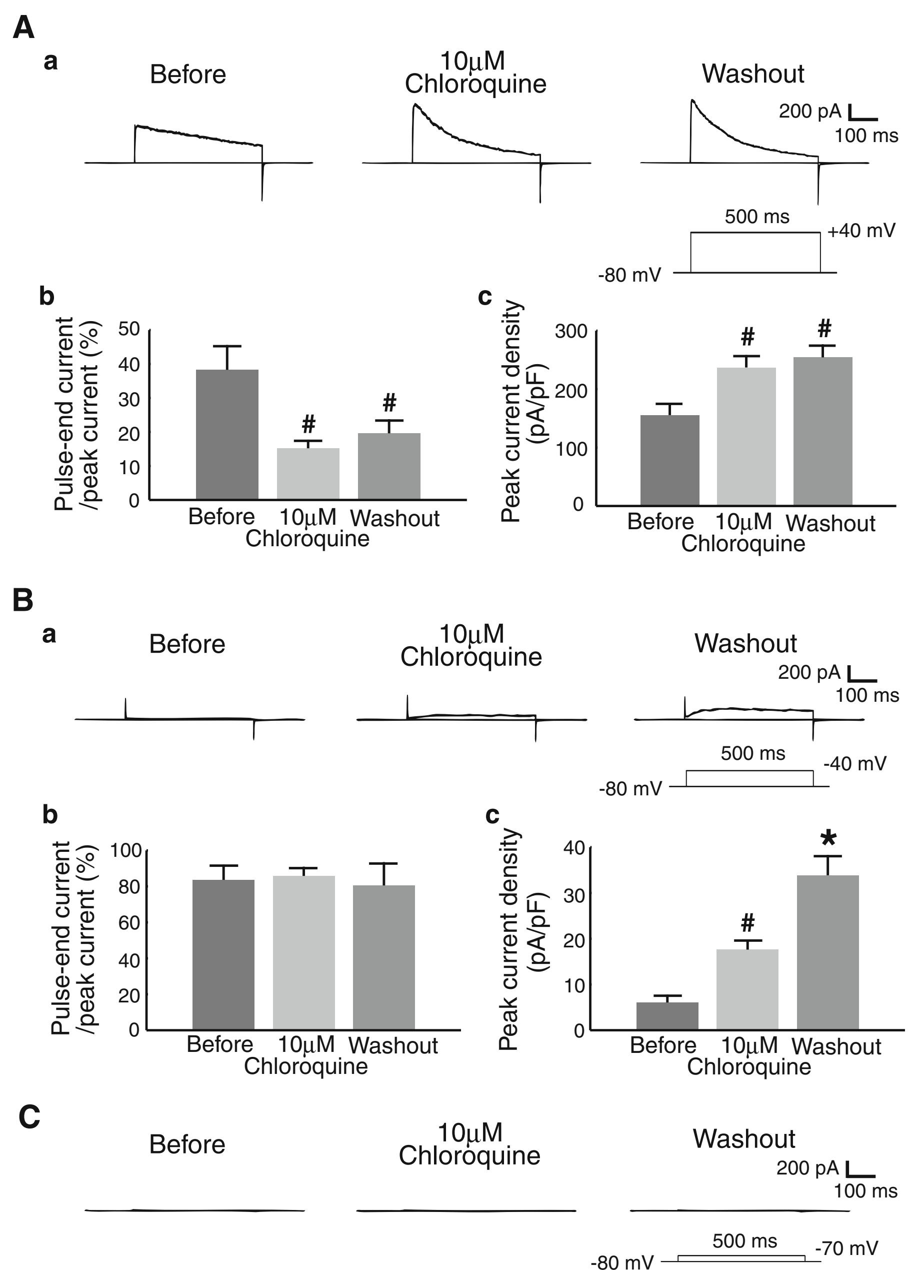

Fig. 2 Effects of chloroquine on Kv1.3-channel currents in murine thymocytes in 500-ms single pulse protocols. Effects of 10 μM chloroquine at the voltage steps of +40 (**A**), −40 (**B**) and −70 mV (**C**). *a* Typical whole-cell current traces recorded before and after the drug application, and after its washout. The currents were elicited from the holding potential of −80 mV to either +40 (**A**), −40 (**B**), or −70 mV (**C**) as depicted in the voltage protocols. Single pulses were applied for 500-ms durations between 1-min intervals. *b* Percentages of the pulse-end currents normalized by the peak currents obtained from the records in *a*. *c* Peak current densities (peak currents normalized by the membrane capacitance). $^{\#}P < 0.05$ versus before chloroquine application. Values are mean ± SEM ($n = 6$). Differences were analyzed by ANOVA followed by Dunnett's or Student's *t* test

effects of chloroquine on these curves (Fig. 4). In a protocol for the activation, stepwise changes in the membrane potential by 10-ms pulses, from the holding potential of −80 mV to the various depolarizing potential levels, evoked membrane currents (Fig. 4A). Fig. 4Aa shows the current density–voltage relationship, demonstrating that the application of chloroquine tends to increase the peak current densities at lower voltgage steps, but not at higher voltgage steps. Then, the peak current densities were divided by the driving force for potassium ions to obtain the conductance. By normalizing each conductance (G) to the maximal conductance (G_{max}), we obtained G/G_{max} to draw activation curves (Fig. 4Ab). The curves were fitted with the Boltzmann equation: $G/G_{max} = 1/\{1 + \exp[(V - V_{1/2})/k]\}$, where V is the membrane potential; $V_{1/2}$, potential for half-maximal activation and k, slope factor. At voltage steps between −40 and +40 mV, chloroquine significantly shifted the curve toward the hyperpolarizing potential changing $V_{1/2}$ from -11.3 ± 1.0 to -22.8 ± 1.6 mV ($n = 6$, $P < 0.05$). Within the range, the increase in G/G_{max} by chloroquine was more emphasized at lower voltgage steps. The result suggests that chloroquine facilitates the activation of the channel currents in a voltage-dependent manner.

On the other hand, in a protocol for the inactivation, stepwise changes in the membrane potential by 500-ms

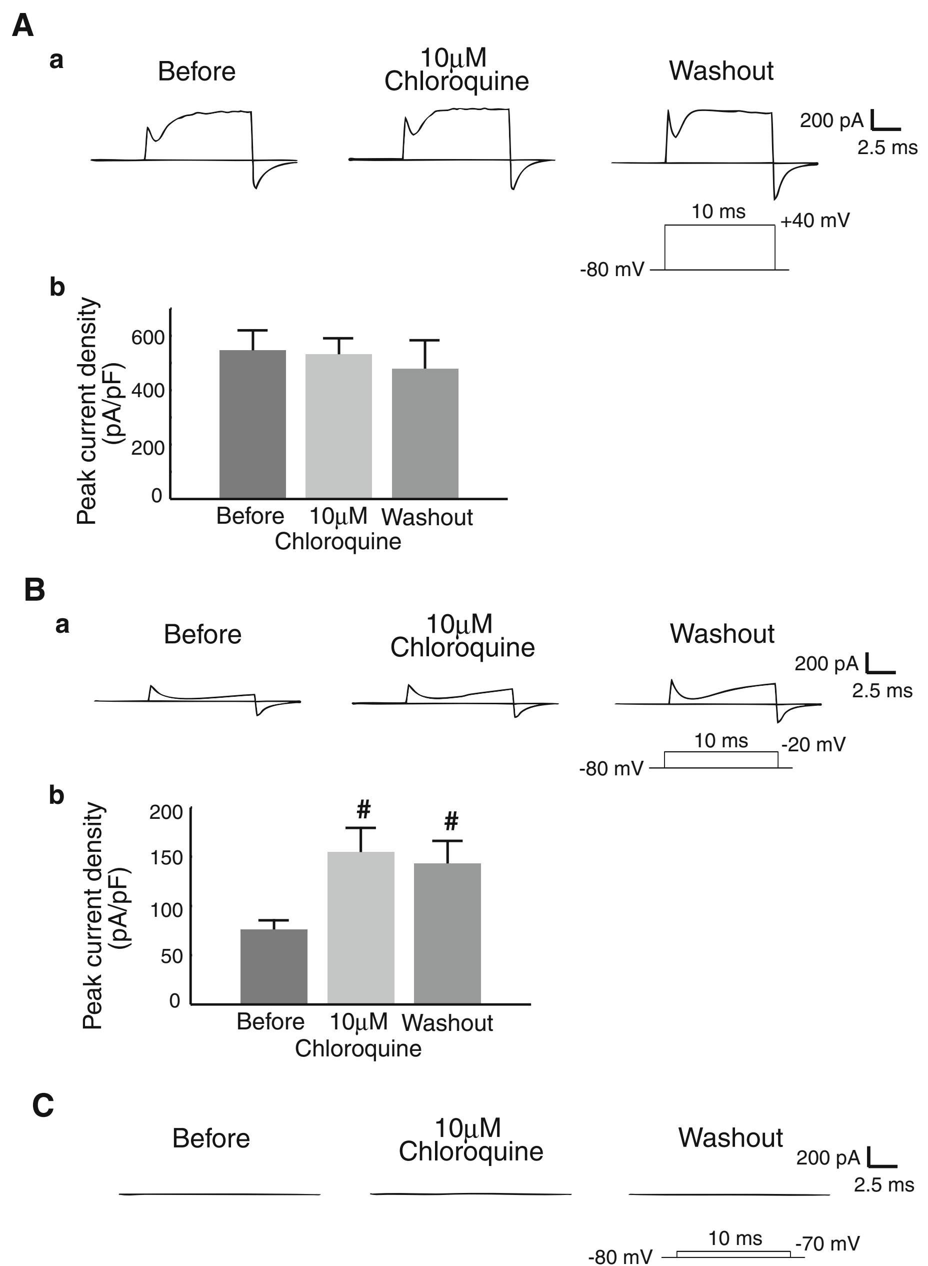

Fig. 3 Effects of chloroquine on Kv1.3-channel currents in murine thymocytes in 10-ms single pulse protocols. Effects of 10 μM chloroquine at the voltage steps of +40 (**A**), −20 (**B**) and −70 mV (**C**). *a* Typical whole-cell current traces recorded before and after the drug application, and after its washout. The currents were elicited from the holding potential of −80 mV to either +40 (**A**), −20 (**B**), or −70 mV (**C**) as depicted in the voltage protocols. Single pulses were applied for 10-ms durations between 5-s intervals. *b* Peak current densities (peak currents normalized by the membrane capacitance). $^{\#}P < 0.05$ versus before chloroquine application. Values are mean ± SEM ($n = 6$). Differences were analyzed by ANOVA followed by Dunnett's or Student's *t* test

conditioning pre-pulses from −80 mV to the various depolarizing potential levels, followed by a 20-ms test pulse of +80 mV, evoked membrane currents (Fig. 4B). By normalizing each peak current (I) to the peak current evoked by pre-pulse (I_0), we obtained I/I_0 to draw inactivation curves (Fig. 4B). The curves were fitted with the Boltzmann equation: $I/I_0 = 1/\{1 + \exp[(V - V_{1/2})/k]\}$, where V is the membrane potential; $V_{1/2}$, potential for half-maximal activation and k, slope factor. Similar to its effects on the activation curves (Fig. 4Ab), chloroquine significantly shifted the curve toward the hyperpolarizing potential ($V_{1/2}$: from −10.1 ± 1.9 to −32.8 ± 7.1 mV, $n = 6$, $P < 0.05$), though the shift occurred in a wide range of voltages. The decrease in I/I_0 by chloroquine was more emphasized at lower voltgage steps. The result suggests that chloroquine also facilitates the inactivation in a voltgage-dependent manner.

Discussion

The major findings from the present study are that chloroquine facilitates both the activation and the inactivation of the channel currents and that those effects are voltgage dependent. Previous studies have demonstrated in cardiomyocytes that chloroquine accelerates the inactivation of

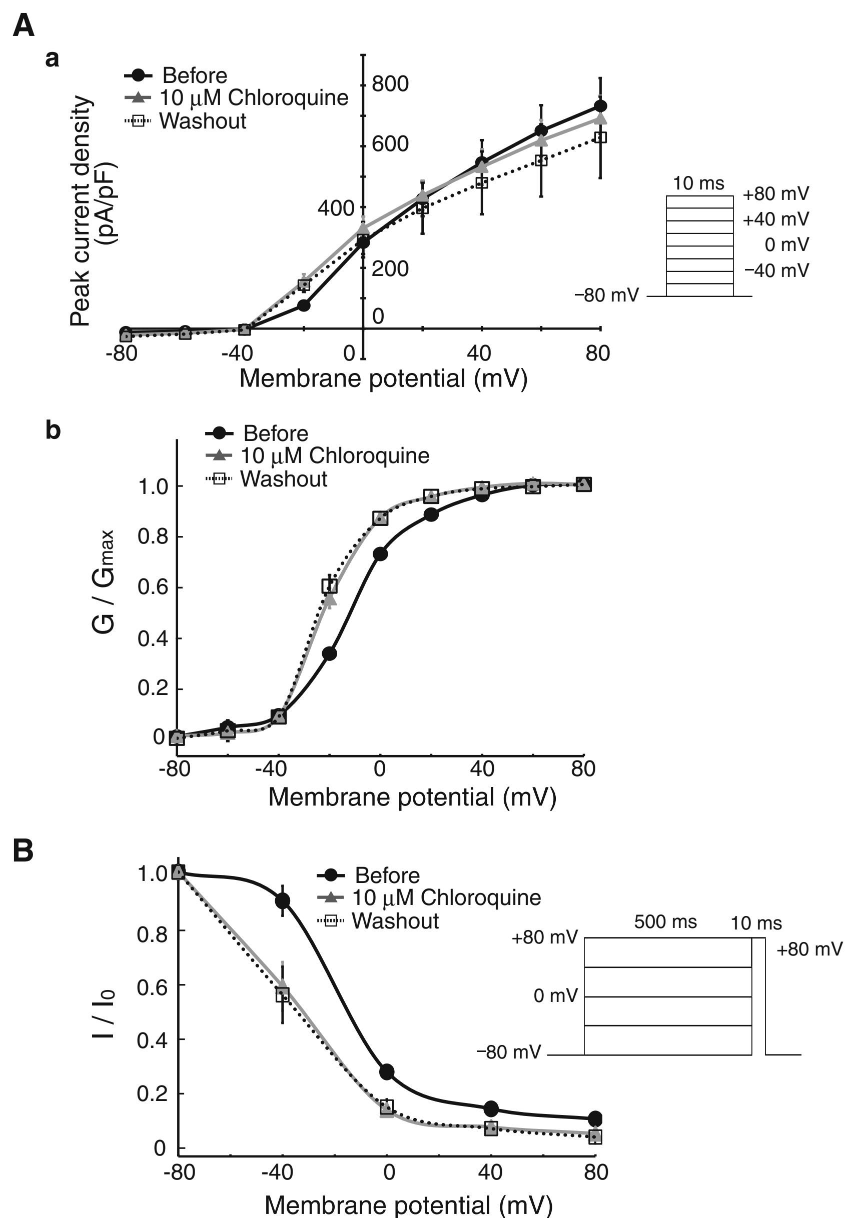

Fig. 4 Effects of chloroquine on the activation and the inactivation kinetics of thymocyte Kv1.3-channel currents. Effects of 10 µM chloroquine on the current density–voltage relationship (**A***a*), the activation curve (**A***b*) and the inactivation curve (**B**). **A** Pulses were applied for 10 ms from the holding potential of −80 to +80 mV in 20-mV increments, while in **B**, a test pulse to +80 mV was applied for 20 ms following 500-ms pre-pulses from −80 to 80 mV in 40-mV increments. They are depicted in the voltage protocols in **A***a* and **B**, respectively. Each point represents the mean ± SEM ($n = 6$)

voltgage-gated K^+ channel currents [9, 10]. However, the effects of chloroquine on the activation have not fully been studied. In the present study, by carefully observing the voltage-dependent differences in the chloroquine's effects, we have clearly shown for the first time that the drug also facilitates the activation of delayed rectifier K^+-channel currents in murine thymocytes.

In the molecular basis, plugging the open-pore of the channels has been considered the mechanism of chloroquine-induced inactivation of the K^+ channel currents [10, 11]. However, this theory alone cannot explain the mechanism of chloroquine-induced activation of the currents observed in the present study. Unveiling the crystal structures of voltage-dependent K^+-channels [12], recent studies have demonstrated that the activation and the inactivation occur by coordinated and sequential openings and closings of the activation and the inactivation gates [14]. The activation gate, comprising the inner transmembrane helix around a gating hinge, opens by an outward bending of the helix, while the inactivation gate, comprising a selectivity filter of the channel, closes by a conformational collapse of the filter. Based on those findings, the following possibilities are considered as to the mechanisms of chloroquine induced activation of the currents. First, as a lipophilic agent that causes the membrane fusion [21], chloroquine may penetrate into the lipid-bilayer of the plasma membrane. Then, it directly perturbs the inner transmembrane helix of the activation gate, bending it

outward to open the gate. Second, as a lysosomal inhibitor [22], chloroquine may protect the channels from lysosomal degradation, and thereby preserves the amount of the channel protein, consequently increasing its density on the plasma membrane.

In 500-ms pulse protocols, chloroquine enhanced the peak currents at both higher and lower voltage steps (Fig. 2Ac, Bc). However, in 10-ms pulse protocols, the drug enhanced the peak currents only at lower (Fig. 3Bb), but not at higher voltage steps (Fig. 3Ab). Based on the results, we can simplify the gating cycles of the channel as depicted in Fig. 5 [13]. Since the long 500-ms pulses generate the equilibria among *C* (closed-state), *O* (opened-state) and *I* (inactivated-state), we can draw a three-state gating model (Fig. 5A). Chloroquine, facilitating both the activation and the inactivation (Fig. 4), accelerates the transitions "$C \rightarrow O$" and "$O \rightarrow I$", and subsequently "$I \rightarrow C$" that represents the recovery from the inactivation (Fig. 5A, circled arrows). On the other hand, the short 10-ms pulses generate the equilibria between *C* and *O* without *I* (Fig. 3Aa, Ba) represented by two-state gating

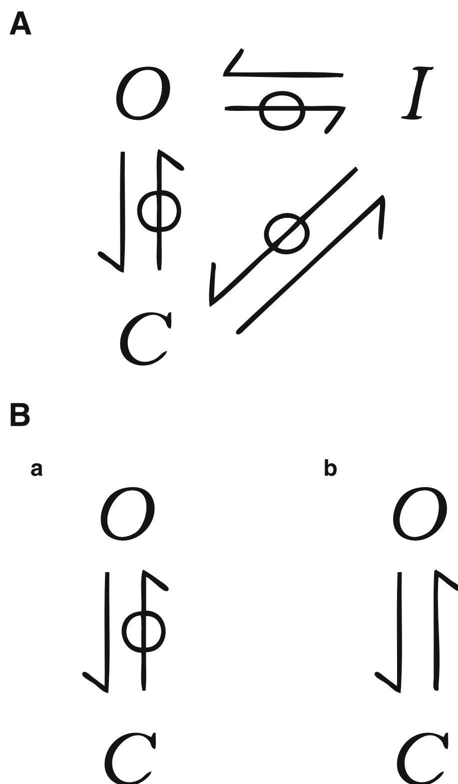

Fig. 5 Simplified gating models of thymocyte Kv1.3-channels. A three-state gating model in 500-ms pulse protocols (**A**) and two-state gating models in 10-ms pulse protocols (**B**). The composite states are C (*closed*), O (*open*) and I (*inactivated*). The transitions "$C \rightarrow O$", "$O \rightarrow I$" and "$I \rightarrow C$" represent the activation, the inactivation and the recovery from the inactivated-state, respectively. *Circled arrows* represent accelerated transitions

models (Fig. 5B). At lower voltage steps, where the equilibrium is *C*-sided due to the low open probability of the channels, chloroquine has enough space to accelerate the transition "$C \rightarrow O$" (Fig. 5Ba, a circled arrow). However, at higher voltage steps, where the equilibrium is *O*-sided due to the high open probability of the channels, chloroquine does not have a space to accelerate the transition any further (Fig. 5Bb). Thus, chloroquine cannot facilitate the activation at higher voltage steps (Fig. 4Ab). As a consequence, the drug did not enhance the peak currents at the higher voltage step in a short pulse protocol (Fig. 3Ab).

K-channels expressed in lymphocytes generate the K^+-diffusion potential across the plasma membranes, and thus play roles in regulating the resting membrane potential and controlling the cell volume [6]. From our results, however, Kv1.3-channels in thymocytes were not activated at the resting membrane potential (Figs. 2C and 3C). This indicates that the channels do not largely contribute to the regulation of the resting membrane potential. In thymocytes, the moderate changes in the transmembrane K^+ gradient generated by the channels were thought to have little effect, compared to the electrogenic activity of Na^+/K^+ pumps [20, 23]. The Kv1.3-channels, however, play crucial roles in initiating the immune response [7, 8]. The membrane hyperpolarization brought about by the opening of the channel triggers the calcium influx necessary for lymphocyte proliferation and IL-2 production. According to previous studies, the complete inhibition of Kv1.3-channel currents [24] or the reduced expression of the channel proteins [25] was involved in the mechanisms of the drug-induced immunosuppression. In the present study, despite its immunosuppressive properties, chloroquine failed to inhibit the channel currents at lower voltage steps that mimic the changes in the membrane potential within the physiological range of thymocytes [20]. However, the large leftward shift of the inactivation curve by chloroquine (Fig. 4B) without the change in the activation threshold at around −40 mV (Fig. 4Ab) implies the reduction of the "activatable" channels at above −40 mV. Such reduction of the channels may be involved in the mechanisms of chloroquine induced immunosuppression in lymphocytes, though additional studies are required to address this issue.

In conclusion, we demonstrated for the first time that chloroquine facilitates both the activation and the inactivation of delayed rectifier K^+-channel currents in thymocytes. We also show that those biphasic effects of chloroquine are voltage-dependent. The direct perturbation of the activation gate was thought to underlie the mechanism of chloroquine-induced activation of the currents.

Conflict of interest The authors declare no conflict of interest.

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