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Block of Kv4.3 potassium channel by trifluoperazine independent of CaMKII

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HIGHLIGHTS

- Trifluoperazine is widely used in the management of schizophrenia.
- Trifluoperazine is known to inhibit Ca^{2+}/calmodulin-dependent phosphorylation.
- Trifluoperazine blocked Kv4.3 by binding to the open state of the channel.
- This effect was not mediated via the inhibition of CaMKII activity.

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ABSTRACT

Trifluoperazine, a trifluoro-methyl phenothiazine derivative, is widely used in the management of schizophrenia and related psychotic disorders. We studied the effects of trifluoperazine on Kv4.3 currents expressed in CHO cells using the whole-cell patch-clamp technique. Trifluoperazine blocked Kv4.3 in a concentration-dependent manner with an IC_{50} value of $8.0 \pm 0.4\,\mu M$ and a Hill coefficient of 2.1 ± 0.1. Trifluoperazine also accelerated the inactivation and activation (time-to-peak) kinetics in a concentration-dependent manner. The effects of trifluoperazine on Kv4.3 were completely reversible after washout. The effects of trifluoperazine were not affected by the pretreatment of KN93, which is another CaMKII inhibitor. In addition, the inclusion of CaMKII inhibitory peptide 281-309 in the pipette solution did not modify the effect of trifluoperazine on Kv4.3. Trifluoperazine shifted the activation curve of Kv4.3 in a hyperpolarizing direction but did not affect the slope factor. The block of Kv4.3 by trifluoperazine was voltage-dependent with a steep increase across the voltage range of channel activation. Voltage dependence was also observed over the full range of activation ($\delta = 0.18$). Trifluoperazine slowed the time course for recovery from inactivation of Kv4.3. Our results indicated that trifluoperazine blocked Kv4.3 by preferentially binding to the open state of the channel. This effect was not mediated via the inhibition of CaMKII activity.

1. Introduction

Trifluoperazine, a phenothiazine derivative, is a widely used conventional antipsychotic drug for the treatment of schizophrenia [1]. While its therapeutic action is ascribed to the blockade of presynaptic D_2 receptors in dopaminergic neurons of the brain [2], trifluoperazine is also known to inhibit the calmodulin-dependent phosphorylation of several protein components, such as sarcoplasmic reticulum proteins [3,4]. There are several reports of trifluoperazine, in its capacity as a CaMKII inhibitor, modulating ion channels in a CaMKII-independent manner. Trifluoperazine inhibited voltage-dependent K^+ channels in rabbit coronary arterial smooth muscle cells and Kv1.3 channels expressed in human T lymphocytes, regardless of its function as a calmodulin inhibitor [5,6]. Trifluoperazine also blocked both HERG channels expressed in *Xenopus* oocytes and the rapidly activating delayed rectifier K^+ currents of guinea pig cardiomyocytes [7]. Kv4.3 underlies the molecular basis of subthreshold activating A-type Kv currents in the

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brain and the smooth muscles, and transient outward Kv currents in the heart [8,9]. Inhibition of CaMKII accelerated the inactivation of transient outward Kv current in human atrial myocytes and in murine colonic myocytes [10,11]. Kv4.3 channels contain three putative sites for CaMKII phosphorylation that are located within the C-terminus [12], and this finding raised the possibility that CaMKII could be a target for Kv4.3 channels and the functional modulation of Kv4.3. Indeed, the phosphorylation of Kv4.3 channels by CaMKII accelerated both the inactivation and the recovery from inactivation of Kv4.3 currents expressed in HEK cells, and the opposite effect was observed with the use of CaMKII inhibitors [12]. However, several studies have alluded to the possibility that CaMKII inhibitors have nonspecific and direct effects on Kv channels. KN93, an inhibitor of CaMKII, reduced the peak amplitude and accelerated the inactivation of Kv4.3 currents in *Xenopus* oocytes [13]. Thus, the present study was designed to investigate whether trifluoperazine directly modulates Kv4.3 in a CaMKII-independent manner using patch-clamp techniques.

2. Materials and methods

We used CHO cells stably expressing Kv4.3L cDNA as described previously [14,15]. The cells were maintained in a humidified atmosphere at 37 °C with 5% CO_2-enriched air. Cells were cultured in Iscove's modified Dulbecco's medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM glutamine (Invitrogen), 0.1 mM hypoxanthine (Invitrogen), 0.01 mM thymidine (Invitrogen), and 0.3 mg/ml G418 (Invitrogen). Kv4.3 currents were recorded using the whole-cell patch-clamp technique with an Axopatch 700B amplifier (Molecular Devices, Sunnyvale, CA, USA) at room temperature (22–24 °C). Patch pipettes were made from borosilicate glass capillaries (PG10165-4, World Precision Instruments, Sarasota, FL, USA) with a micropipette puller. 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 using KOH. 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 using NaOH. Trifluoperazine (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was dissolved in distilled water and KN93 (Santa Cruz Biotechnology, Inc.) was dissolved in DMSO (Sigma, St. Louis, MO, USA). CaMKII inhibitory peptide 281-309 (Calbiochem, La Jolla, CA, USA) was dissolved in the internal pipette solution. Analysis of the data was performed using pClamp 10.0 software (Molecular Devices) and Origin 8.0 software (Microcal Software, Inc., Northampton, MA, USA). The results are expressed as the mean ± S.E. A paired Student's *t*-test and an analysis of variance for comparisons of multiple groups followed by a Bonferroni's test were used for the statistical analyses. A value of $P < 0.05$ was considered statistically significant.

3. Results

Fig. 1 A shows the whole-cell recordings of Kv4.3 currents elicited by 500-ms depolarizing pulses to +40 mV from a holding potential of −80 mV every 10 s in the absence and presence of trifluoperazine. Trifluoperazine slightly decreased the peak amplitude, but significantly accelerated the inactivation of Kv4.3 in a concentration-dependent manner, so that the block of Kv4.3 currents was measured as a reduction in the integral of the current during depolarization. The blockade measured on the integral was fitted using the Hill equation and yielded an IC_{50} value of 8.0 ± 0.4 μM and a Hill coefficient of 2.1 ± 0.1 ($n = 7$) (Fig. 1B). The concentration-response curve for Kv4.3 was rather steep with a Hill coefficient of 2.1, which suggested that there may have been more than one binding site for trifluoperazine or that the binding was positively cooperative [16]. The time course for the decay of Kv4.3 at +40 mV under control conditions was fitted with a biexponential function with a fast time constant of 31.3 ± 1.5 ms, and a slow time constant of 145.3 ± 8.8 ms ($n = 7$) (Fig. 1C). In the presence of trifluoperazine, the time course for the decay of Kv4.3 was also fitted with a biexponential function. Trifluoperazine decreased both the fast and slow time constants in a concentration-dependent manner, suggesting an acceleration of Kv4.3 inactivation kinetics. To investigate the effect on the activation kinetics, we measured the time-to-peak of Kv4.3 currents (Fig. 1D). The time-to-peak of Kv4.3 activation was significantly reduced for all concentrations tested. These accelerations in the decay rate and activation time courses of Kv4.3 suggest open-channel block mechanisms [17,18].

Fig. 2A and B show the time course of changes in the integral of Kv4.3 currents during the repeated application of trifluoperazine. Bath application of trifluoperazine induced a rapid acceleration of Kv4.3 inactivation, thus decreasing the integral of Kv4.3 currents. This inhibition appeared within 20 s after application of the drug, and reached a steady-state within 4 min. The Kv4.3 current was decreased to 49.7 ± 1.5% ($n = 7$) of the control value. The effect of trifluoperazine on Kv4.3 could be largely and repeatedly recovered after a 4-min washout of the drug-free bath solution (restored to 94.3 ± 4.1% ($n = 7$) of its control value). These effects were also observed repeatedly. To rule out the possibility of the involvement of CaMKII inhibition by trifluoperazine, we used other CaMKII inhibitors, KN93 and CaMKII inhibitory peptide 281-309. The addition of KN93 in the bath solution decreased Kv4.3 by 20.4 ± 1.2% ($n = 6$) (Fig. 2C). The subsequent application of trifluoperazine further reduced Kv4.3 by 61.2 ± 3.0% ($n = 6$). The dialysis of cells with CaMKII inhibitory peptide for 5 min had no effect on the peak or kinetics of Kv4.3 (108.1 ± 5.8% ($n = 7$) of the control value) (Fig. 2D). Application of trifluoperazine significantly decreased Kv4.3 by 54.4 ± 5.6%. Since KN93 and CaMKII inhibitory peptide have a potent inhibitory effect on CaMKII phosphorylation activity, and the concentrations of the inhibitors used in this study were sufficiently high to inhibit CaMKII activity completely [19], our results suggested that trifluoperazine affected Kv4.3 in a CaMKII-independent manner.

Fig. 3A and B show the representative Kv4.3 currents elicited after applying 500 ms depolarizing pulses from a holding potential of −80 mV to +60 mV in 10-mV steps every 10 s in the absence and presence of trifluoperazine. Trifluoperazine reduced the amplitude of Kv4.3 currents at all membrane potentials tested. Fig. 3C shows the plots of normalized conductance (G/G_{max}) versus test potential. The data were fitted using a Boltzmann equation. The activation curve of Kv4.3 gave the half-activation potential ($V_{1/2} = -4.7 \pm 0.9$) and the slope factor ($k = 15.7 \pm 0.1$, $n = 7$) under control conditions. Trifluoperazine slightly shifted the activation curve of Kv4.3 in a hyperpolarizing direction ($V_{1/2} = -7.8 \pm 1.2$, $k = 16.8 \pm 0.8$, $n = 7$, $P < 0.05$), but did not affect the slope factor. To evaluate the voltage dependency of the block by trifluoperazine, the fractional block of the integral ($I_{Trifluoperazine}/I_{Control}$) was plotted against the membrane potential (Fig. 3D). The block was steeply increased in a voltage range that coincided with the activation of Kv4.3 ($F_{4,30} = 7.33$, $P < 0.01$). At more depolarized potentials where the channels were fully activated (between +20 and +60 mV), the trifluoperazine block was also voltage-dependent. This voltage dependence was fitted with a Woodhull's equation and yielded $\delta = 0.18 \pm 0.05$ ($n = 7$).

The cell was depolarized to +40 mV for 500 ms and then repolarized for a variable period before applying the same pulses to assess the extent of recovery from inactivation of Kv4.3 in the absence and presence of trifluoperazine (Fig. 4). Under control conditions, recovery from inactivation could be well fitted with a single exponential function with a time constant of 217.5 ± 23.1 ms ($n = 6$) (Fig. 4B). In the presence of trifluoperazine, the time course of recovery

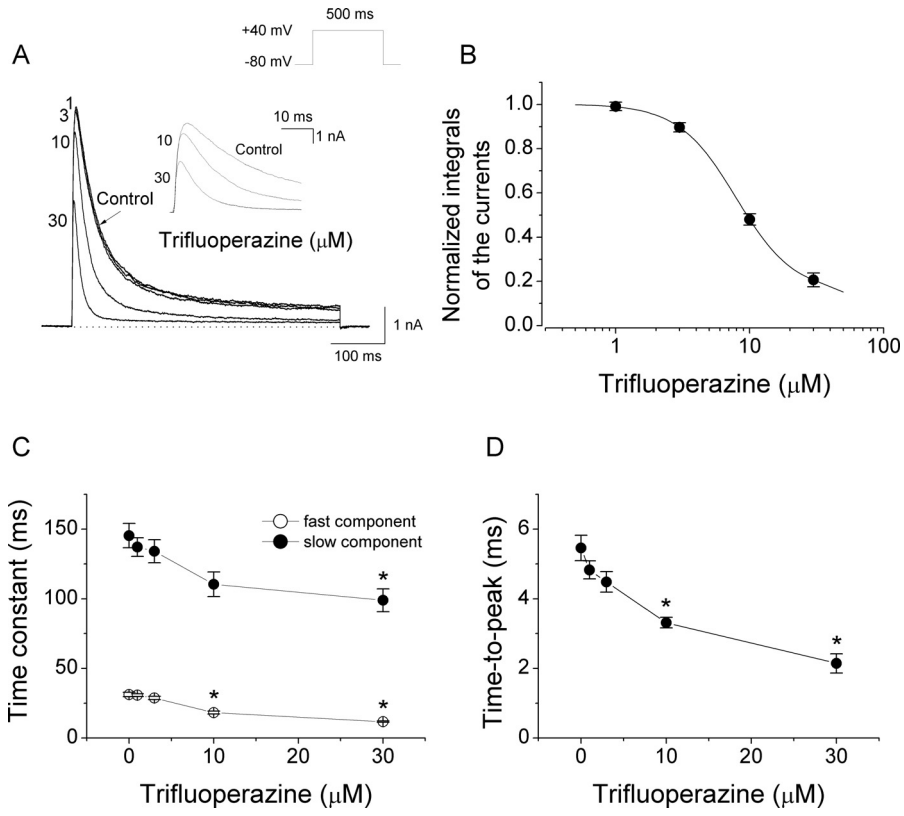

Fig. 1. (A) Whole-cell Kv4.3 currents were elicited by a 500-ms depolarizing pulse to +40 mV from a holding potential of −80 mV every 10 s. The effects of 1, 3, 10, and 30 μM of trifluoperazine are shown. The dotted line marks zero current. The inset shows the Kv4.3 currents on an expanded time scale for the first 50 ms of the traces. (B) Normalized blocks of the integral of Kv4.3 currents during depolarization are plotted as a function of trifluoperazine concentrations. Data were fitted with the Hill equation. (C) Inactivation kinetics and (D) time-to-peak of Kv4.3. The fast and slow components of time constants and time-to-peak as a function of the concentration of trifluoperazine are shown. *$P < 0.05$, significant difference from the control. Data are expressed as the mean ± S.E.

from inactivation of Kv4.3 was fitted with a biexponential function with a fast time constant of 250.6 ± 25.8 ms and a slow time constant of 1235.9 ± 138.8 ms ($n = 6$, $P < 0.05$), suggesting that trifluoperazine significantly delayed the recovery from inactivation of Kv4.3.

4. Discussion

Kv4.3 is a membrane of the *Shal*-type Kv channels and is widely expressed in a variety of tissues, such as the brain, heart and smooth muscles [15]. The primary sequence of Kv4.3 channels contains three consensus sites for phosphorylation by CaMKII [12]. Accordingly, several studies have reported a modulation of the functional activity of Kv4.3 channels by CaMKII. For example, autothiophosphorylated CaMKII decreased the rate of the inactivation and accelerated the recovery from inactivation of Kv4.3 currents expressed in HEK cells [12]. CaMKII inhibitors resulted in an opposite effect, suggesting that CaMKII modulates the kinetics of Kv4.3 by direct phosphorylation [12]. In the present study, trifluoperazine slightly decreased the peak amplitude of Kv4.3 but accelerated the apparent inactivation rate and the time course of activation (time-to-peak). These characteristics of Kv4.3 block were similar to an open-channel block [18,20]. Moreover, the block of Kv4.3 increased with depolarization, indicating a voltage-dependent block. These results also indicated preferential binding of the drug to the open state of Kv4.3 channels [21]. In addition, our data suggest that these inhibitory properties are not mediated via the inhibition of CaMKII, but are probably due to a direct interaction of the drug with Kv4.3 channels. In the present study, the time course of trifluoperazine wash-in and wash-out was rapid, and evidently reflected the direct interaction between the drug and the channel for a block of Kv4.3. Although the Kv4.3 channel is known to be phosphorylated under resting conditions with low intracellular Ca^{2+} concentrations [22], the inclusion of CaMKII inhibitory peptide in pipette solution had no effect on the kinetics of Kv4.3 up to 5 min. Furthermore, the presence of CaMKII inhibitory peptide in the pipette solutions or KN93 in the bath solution did not prevent the inhibitory properties of trifluoperazine on Kv4.3 currents. Indeed, several inhibitors of CaMKII have been reported to block different voltage-dependent ion channels without mediation through CaMKII inhibition. For example, KN93, a potent inhibitor of CaMKII, reduced the peak currents and accelerated the inactivation of Kv4.3 expressed in *Xenopus* oocytes, suggesting direct interaction with the channel pore [13]. Trifluoperazine caused a concentration-, time-, and voltage-dependent block of Kv1.3, suggesting that the blocking effect of the drug was CaMKII-independent and was due to a direct effect on Kv1.3 channels [6]. Trifluoperazine also inhibited Nav1.7 and Nav1.4 sodium channels in a state-dependent manner, which did not involve a disruption of CaMKII–channel interactions [23]. KN93 and KN92, its inactive analog, reversibly inhibited Cav1.3 and Cav1.2 calcium channels in a CaMKII-independent manner [24]. However, autothiophosphorylated CaMKII decreased the rate of Kv4.3 inactivation expressed in HEK cells and CaMKII inhibitors resulted in the opposite effect, suggesting the direct phosphorylation of Kv4.3 by CaMKII [12]. We cannot completely rule out the possibility that the effect of trifluoperazine on Kv4.3 occurred via the inhibition of CaMKII in our study. Alternatively, one possible explanation could be that this difference may have reflected the coassembly of β-subunits that were differentially expressed in these two cell lines [25].

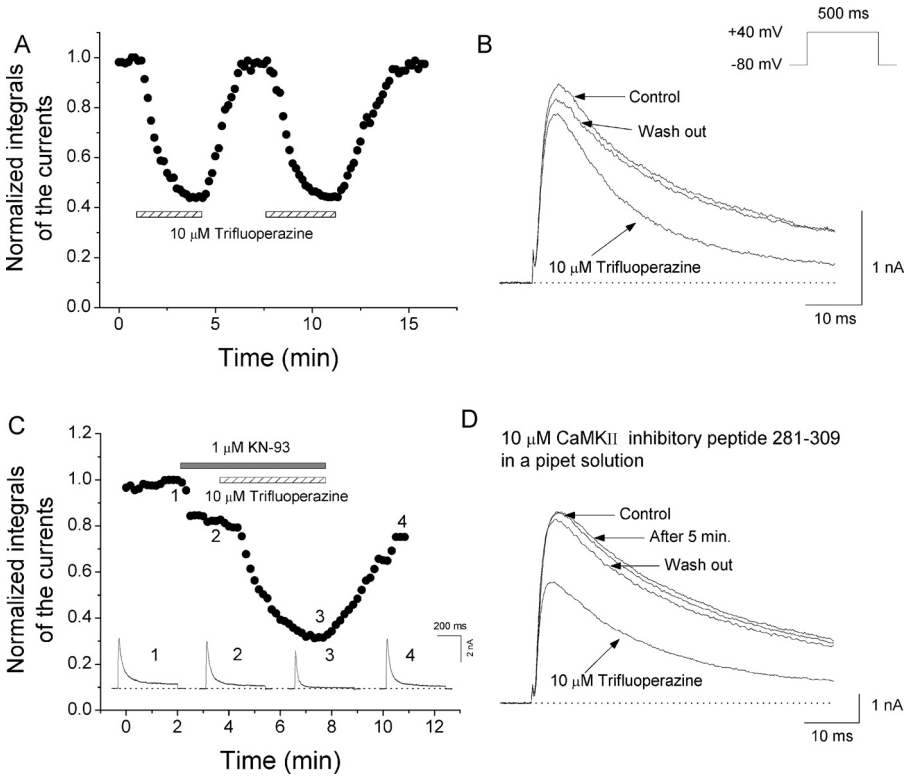

Fig. 2. (A) Reversible inhibition of Kv4.3 by trifluoperazine. Kv4.3 currents were elicited by a 500-ms depolarizing pulse to +40 mV from a holding potential of −80 mV every 10 s. Normalized blocks of the integral of Kv4.3 current during depolarization are plotted as a function of time. (B) The current traces of Kv4.3 are shown in the absence and presence of trifluoperazine, and after washout of the drug. (C) The Kv4.3 current traces recorded under control conditions, and in the presence of KN93 and trifluoperazine. Normalized blocks of the integral of Kv4.3 current during depolarization are plotted as a function of time. (D) CaMKII inhibitory peptide 281-309 was included in the pipette solution. Control current was recorded immediately after membrane rupture, also shown are the current recorded 5 min after whole-cell recording, and the current measured after treatment with trifluoperazine and washout of the drug. The dotted line marks zero current.

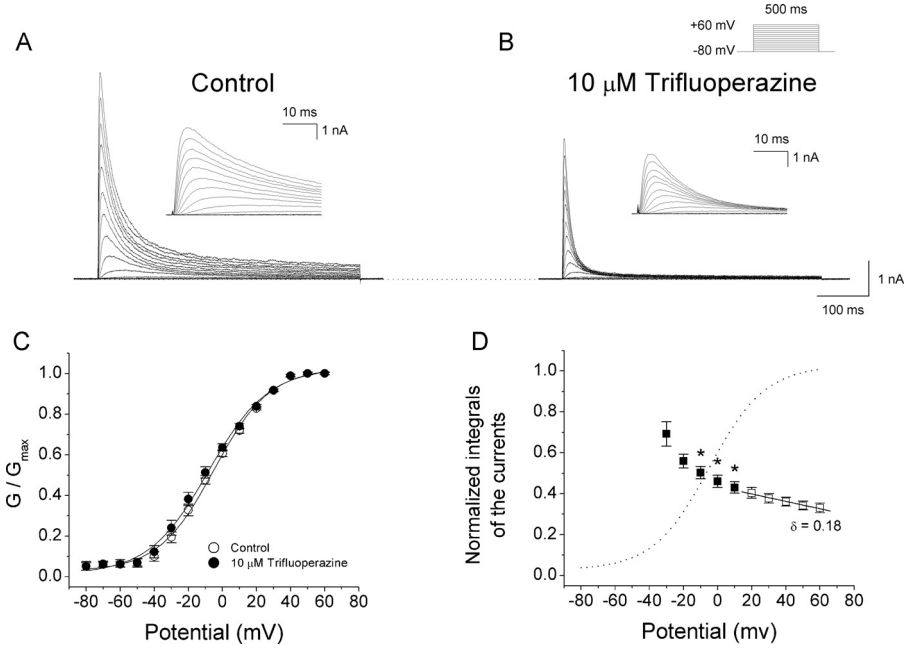

Fig. 3. The whole-cell Kv4.3 currents in the absence (A) and in the presence of trifluoperazine (B). The inset shows the first 50 ms of the current recordings on an expanded time scale. The dotted line marks zero current. (C) The plots of normalized conductance (G/G_{max}) versus test potential. The conductances (G) were calculated by dividing the peak amplitude of Kv4.3 at the test potential by assuming a reversal potential of −85 mV under the ionic conditions of our experiment. The maximal conductance (G_{max}) was obtained by a fitting of the normalized data. The data were fitted using a Boltzmann equation. (D) The integral current of Kv4.3 in the presence of trifluoperazine was normalized to that of the control at each voltage. The dashed line represents the activation curve of Kv4.3 under control conditions. The solid lines represent the linear fit. Data are expressed as the mean ± S.E.

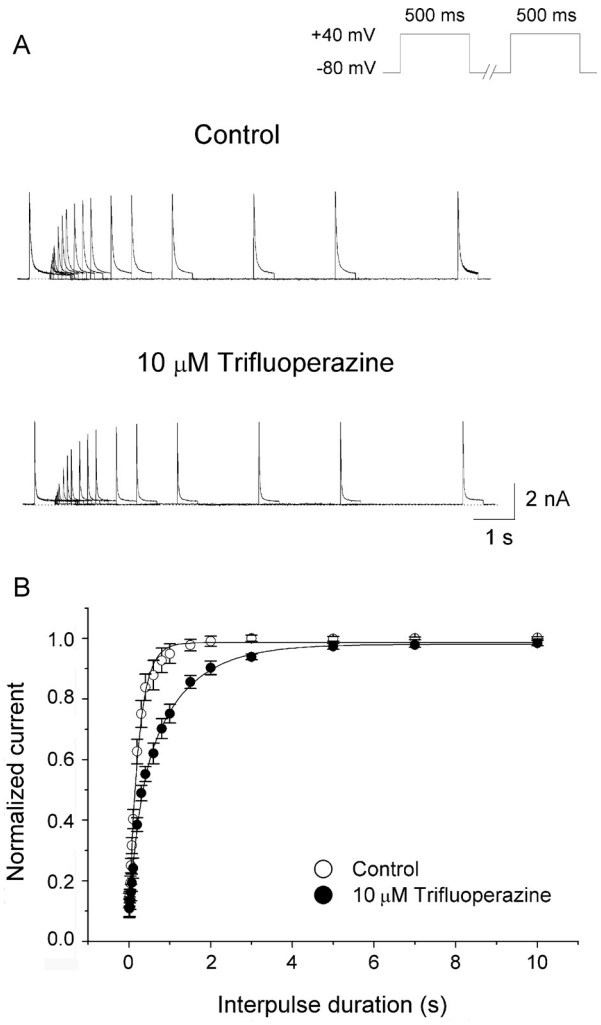

Fig. 4. (A) Representative current traces of Kv4.3 were recorded using double-pulse protocols in the absence and presence of trifluoperazine. (B) Time course of recovery from inactivation of Kv4.3 was fitted with a single exponential function under control conditions and was fitted with a biexponential function in the presence of trifluoperazine. Data are expressed as the mean ± S.E.

Trifluoperazine is a trifluoro-methyl phenothiazine derivative and is widely used in the management of schizophrenia and related psychotic disorders [1]. Besides inhibition of calmodulin-dependent phosphorylation, trifluoperazine has the blocking action of postsynaptic D_2 receptors in dopaminergic neurons, which is attributed to initiating the antipsychotic therapeutic actions of this drug [2]. Kv4.3 underlies somatodendritic A-type Kv currents and is highly expressed in dopaminergic neurons in the brain [26]. Indeed, haloperidol, another antipsychotic, increased Kv4.3 current density and altered intrinsic neuronal excitability in rat dopaminergic neurons [27]. In this context, there are considerable possibilities that some aspects of the therapeutic and/or adverse effects of trifluoperazine in the CNS are mediated by its action on Kv4.3 channels. The therapeutic plasma concentrations of trifluoperazine range from 0.001 to 0.01 μg/ml (equivalent to 2–20 nM) in patients using trifluoperazine [28]. In the present study, trifluoperazine blocked Kv4.3 with an IC_{50} of 8.0 μM, which was much higher than the therapeutic plasma concentrations. Under certain conditions, however, the concentrations of trifluoperazine in the blood can reach as high as micromolar concentrations [29]. Thus, the inhibitory effect of trifluoperazine on Kv4.3 may be clinically relevant in the upper ranges of therapeutic plasma concentrations that can occur under various clinical situations. It should be noted, however, that Kv4.3 encodes a pore-forming and voltage-sensing α-subunit and co-assembles with ancillary β-subunits including KChIP, which modulate kinetic properties, drug sensitivity, and the surface expression of Kv4.3 channels in native cells [30].

The present study has demonstrated that trifluoperazine reversibly produced a concentration- and voltage-dependent block of Kv4.3. These results suggest that trifluoperazine did not produce its effects through the inhibition of intracellular CaMKII but directly interacted with the open state of Kv4.3 channels.

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