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The calmodulin inhibitor and antipsychotic drug trifluoperazine inhibits voltage-dependent K^+ channels in rabbit coronary arterial smooth muscle cells

Da Hye Hong [a], Youn Kyoung Son [a], Hongliang Li [a], In Duk Jung [b], Yeong-Min Park [b], Won-Kyo Jung [c], Han Sol Kim [d], Il-Whan Choi [e,1,*], Won Sun Park [a,1,*]

[a] Department of Physiology, Kangwon National University School of Medicine, Chuncheon 200-701, South Korea
[b] Department of Immunology, Lab of Dendritic Cell Differentiation and Regulation, College of Medicine, Konkuk University, Chungju 380-701, South Korea
[c] Department of Biomedical Engineering, and Center for Marine-Integrated Biomedical Technology (BK21 Plus), Pukyong National University, Busan 608-737, South Korea
[d] Department of Bio-health Technology, Kangwon National University, Chuncheon 200-701, South Korea
[e] Department of Microbiology, Inje University College of Medicine, Busan 614-735, South Korea

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ABSTRACT

We investigated the effect of the calmodulin inhibitor and antipsychotic drug trifluoperazine on voltage-dependent K^+ (Kv) channels. Kv currents were recorded by whole-cell configuration of patch clamp in freshly isolated rabbit coronary arterial smooth muscle cells. The amplitudes of Kv currents were reduced by trifluoperazine in a concentration-dependent manner, with an apparent IC_{50} value of 1.58 ± 0.48 μM. The rate constants of association and dissociation by trifluoperazine were 3.73 ± 0.33 $\mu M^{-1}\ s^{-1}$ and 5.84 ± 1.41 s^{-1}, respectively. Application of trifluoperazine caused a positive shift in the activation curve but had no significant effect on the inactivation curve. Furthermore, trifluoperazine provoked use-dependent inhibition of the Kv current under train pulses (1 or 2 Hz). These findings suggest that trifluoperazine interacts with Kv current in a closed state and inhibits Kv current in the open state in a time- and use-dependent manner, regardless of its function as a calmodulin inhibitor and antipsychotic drug.

1. Introduction

Calmodulin (calcium-modulated protein) functions as multifunctional intermediate messenger protein by binding calcium ions and is involved in inflammation, metabolism, apoptosis, smooth muscle contraction, and intracellular movement [1]. Calmodulin acts as a calcium sensor and signal transducer and is associated with various intracellular signaling systems. Phenothiazines are well-known calmodulin inhibitors widely used as antipsychotic agents. Trifluoperazine is a representative antipsychotic drug classified in the phenothiazine group and is mainly used to treat schizophrenia [2]. Although trifluoperazine is efficacious as an antipsychotic agent, its use has decreased steadily. Administration of trifluoperazine commonly causes side effects, including drowsiness, dizziness, rash, and anorexia [3]. It may also cause temporary dystonia (difficulty walking or moving) [4]. However, the largest problem is that trifluoperazine has the potential to cause tardive dyskinesia, an extrapyramidal symptom [2,5]. Despite the many side effects of trifluoperazine, many questions remain about its effect on vascular ion channels.

Four broad types of K^+ channels have been confirmed in vascular smooth muscle cells: voltage-dependent K^+ (Kv), big conductance Ca^{2+}-activated K^+ (BK_{Ca}), ATP-sensitive K^+ (K_{ATP}), and inwardly rectifying K^+ (Kir) channels [6,7]. Among these, Kv channels play an important role in maintaining basal tone by controlling resting membrane potential [6,8,9]. Inhibition of Kv channels induces membrane depolarization and vasoconstriction in some arteries, which are closely associated with vascular dysfunctions including hypertension, hypoxia, diabetes, and cardiac diseases [10]. Thus, modulating Kv channel functions is a potential therapeutic target to overcome vascular diseases.

Considering the efficacy of trifluoperazine as a calmodulin inhibitor and antipsychotic, and the physiological relevance of vascular Kv channels, the action of trifluoperazine on native vascular Kv channels should be established to elucidate its vascular toxic effects upon application to vascular smooth muscle.

In the present study, we investigated the effect of trifluoperazine on Kv channels in freshly isolated coronary arterial smooth muscle cells using the patch clamp technique. We showed that

* Corresponding authors. Fax: +82 51 891 6004 (I.W. Choi). Address: Department of Physiology, Kangwon National University School of Medicine, 1 Kangwondaehak-gil, Chuncheon 200-701, South Korea. Fax: +82 33 255 8809 (W.S. Park).
E-mail addresses: cihima@inje.ac.kr (I.-W. Choi), parkws@kangwon.ac.kr (W.S. Park).
[1] These authors contributed equally to this work.

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trifluoperazine blocks Kv channels in state-, time-, and use-dependent fashions, regardless of its roles as a calmodulin inhibitor and antipsychotic agent.

2. Materials and methods

2.1. Single cell isolation

Male New Zealand White rabbits (weight, 2.0–2.5 kg) were sacrificed using a Zoletil (15 ml/kg)/Rompun (0.5 ml/kg) mixture, which were injected into the hind limb muscle. The Committee for Animal Experiments of Kangwon National University approved the use of rabbits. The left descending coronary arteries were isolated and cleaned of connective tissue in normal Tyrode's solution. We carried out a two-step enzyme treatment with papain (1.0 mg/ml) and collagenase (2.8 mg/ml) in 1 ml of Ca^{2+}-free normal Tyrode's solution to obtain single coronary smooth muscle cells. The two enzymes contained the same concentration of bovine serum albumin (1.0 mg/ml), and dithiothreitol (1.0 mg/ml). The arteries were incubated for 25 or 21–22 min at 37 °C, respectively. After the incubation, the arteries were agitated using a fire-polished Pasture pipette. The isolated cells were maintained at 4 °C in Kraft-Brühe (KB) solution and used within 10 h.

2.2. Solutions and chemicals

The bath solution (normal Tyrode's solution) contained (mM): NaCl, 135; KCl, 5.4; NaH_2PO_4, 0.33; $CaCl_2$, 1.8; $MgCl_2$, 0.5; HEPES, 5; glucose, 16.6; adjusted to pH 7.4 using NaOH. The KB solution contained (mM): KOH, 70; L-glutamate, 50; KH_2PO_4, 20; KCl, 55; taurine, 20; $MgCl_2$, 3; glucose, 20; HEPES, 10; EGTA, 0.5; adjusted to pH 7.3 with KOH. The pipette-filling solution contained (mM): K-aspartate, 110; KCl, 25; NaCl, 5; $MgCl_2$, 1; Mg-ATP, 4; EGTA, 10; HEPES, 10; adjusted to pH 7.2 with KOH. Trifluoperazine was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and dissolved in distilled water.

2.3. Electrophysiology and data analysis

We recorded whole-cell Kv currents from coronary arterial smooth muscle cells using a digital interface, (NI-DAQ-7; National Instruments, Union, CA, USA) and amplifier (EPC-8; Medical System Corp., Darmstadt, Germany). The pulse protocol was generated by PatchPro software. The patch pipettes were fabricated from borosilicate capillaries (Clark Electromedical Instruments, Pangbourne, UK) using a PP-830 vertical puller (Narishige Scientific Instrument Laboratory, Tokyo, Japan). The patch pipette had a resistance of 3–4 MΩ when filled with the internal solution. Data acquisition was performed at a sampling rate of 1–3 kHz and filtered at 0.5–1.0 kHz for the whole-cell configuration.

We used the Origin 7.0 software (Microcal Software, Inc., Northampton, MA, USA) for data analysis. As described previously [11], the interaction kinetics between the drug and channel were expressed as a first-order reaction. The IC_{50} value and the Hill coefficient (n) were obtained through concentration-dependent data fitting to the following Hill equation:

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

where f is fractional inhibition ($f = 1 - I_{drug}/I_{control}$) at the test potential, and [D] is drug concentration.

The activation kinetics was fitted with a single exponential function to determine the dominant time constant of the activation process. The inactivation time courses of the currents were fitted with a single (control condition) or double (trifluoperazine) exponential function. The apparent rate constants for association (k_{+1}) and dissociation (k_{-1}) were obtained with the following equations:

$$1/\tau_D = k_{+1}[D] + k_{-1},$$

$$K_d = k_{-1}/k_{+1},$$

where τ_D means the time constant of drug-induced inhibition.

The experimental current values (I) were calculated as:

$$\text{Normalized } I = (I - I_c)/(I_{max} - I_c),$$

where I_{max} represents the measured maximum current and I_c represents a nonzero current that was not inactivated. We eliminated nonzero residual current from the actual values.

Activation curves were derived from the tail current elicited by returning to −40 mV after a depolarizing pulse. Activation curves were fitted with the Boltzmann equation:

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

where k represents the slope factor, V is the test potential, and $V_{1/2}$ is the half-maximal activation point.

Steady-state inactivation data were acquired from a two-pulse protocol; currents were measured with a 600-ms test potential to +40 mV after 7 s preconditioning pulses of −80 to +30 mV intervals of 10 mV in the absence and presence of the drug. The steady-state inactivation curves were fitted with another Boltzmann equation.

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

where V is the test potential, $V_{1/2}$ means the potential at the midpoint of maximum inactivation, and k represents the slope factor of the curve.

Results are presented as means ± standard errors. Student's t-test was used to test for statistical significance. A value of $P < 0.05$ was taken to indicate statistical significance.

3. Results

3.1. Trifluoperazine-induced inhibition of the Kv current

We recorded the Kv current using whole-cell patch clamp technique to assess the effect of trifluoperazine on rabbit coronary arterial smooth muscle cells. The other K^+ currents, such as K_{ATP} and BK_{Ca} channels were effectively excluded by the inclusion of ATP and EGTA, respectively, in the pipette solution. Fig. 1 shows the inhibitory effect of 3 μM trifluoperazine on Kv currents recorded with depolarizing step pulses from −60 to +60 mV in steps of 10 mV with a holding potential of −60 mV. As shown in Fig. 1A, the Kv current was rapidly activated and then slowly and partially inactivated (intrinsic inactivation). The effects of 3 μM trifluoperazine are illustrated in Fig. 1B. The Kv current decreased when cells were exposed to 3 μM trifluoperazine, and this inhibition occurred rapidly (within 2 min) and was partially washed out (data not shown). In the absence and presence of 3 μM trifluoperazine, the analysis of the peak and steady-state current–voltage (I–V) relationships showed that trifluoperazine predominantly inhibited the steady-state Kv current rather than the peak current (Fig. 1C and D).

3.2. Trifluoperazine inhibits the Kv current in a concentration-dependent manner

Fig. 2A shows the dose-dependent inhibition of Kv currents by various trifluoperazine concentrations (0, 0.1, 0.3, 1, 3, 10, and 30 μM). The trifluoperazine-induced inhibition of Kv currents measured at peak and steady-state are summarized in

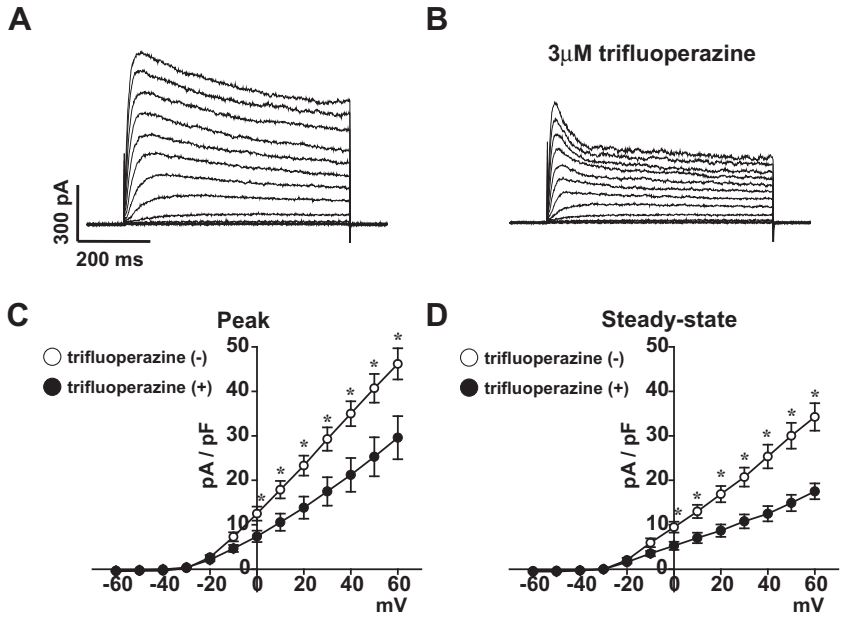

Fig. 1. Effects of trifluoperazine on voltage-dependent K^+ (Kv) channels in rabbit coronary arterial smooth muscle cells. Kv currents were elicited by 600-ms depolarizing pulses between –60 and +60 mV at 10-mV increments of –60 mV under control conditions (A) and in the presence of 3 μM trifluoperazine (B). Current–voltage (*I–V*) relationship of peak (C) and steady-state (D) Kv currents in the absence (○) or presence (●) of trifluoperazine. $n = 5$. $^*P < 0.05$.

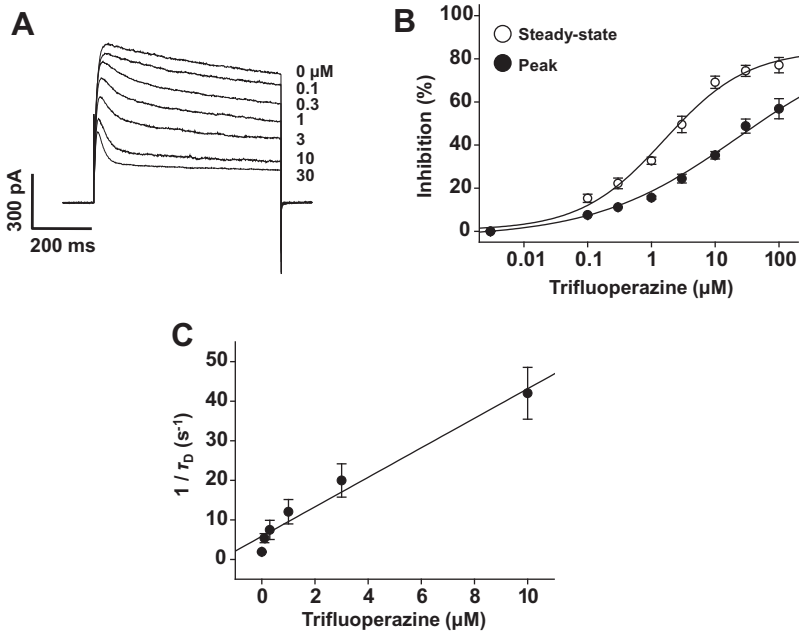

Fig. 2. Dose-dependent inhibition Kv current by trifluoperazine. (A) Representative current traces were elicited by 600-ms one-step depolarizing pulses from a holding potential of –60 mV to +60 mV in the presence of 0, 0.1, 0.3, 1, 3, 10, and 30 μM trifluoperazine. (B) The percentage inhibitions of the Kv current measured at peak (●) and steady-state (○) are plotted against trifluoperazine concentration. Normalized currents were fitted to the Hill equation. All $n = 6$. (C) Time constants (τ_D) were obtained by single (absence of trifluoperazine) and double (presence of trifluoperazine) exponential functions using the decay phase of the traces in Fig. 2A. Data are plotted as a reciprocal of τ_D at +60 mV against the various trifluoperazine concentrations ($n = 6$). The apparent association (k_{+1}) and dissociation rate constants (k_{-1}) were calculated from $1/\tau_D = k_{+1}[D] + k_{-1}$.

Fig. 2B. As described in Fig. 1, the degree of inhibition of the Kv current by trifluoperazine was greater for the steady-state current than that for the peak current. For steady-state inhibition, a nonlinear least-squares fit of the Hill equation to the concentration–response data at +60 mV produced an apparent IC_{50} value of 1.58 ± 0.48 μM and a Hill coefficient of 0.66 ± 0.13 (Fig. 2B).

3.3. Concentration dependence of the time course of Kv current inhibition by trifluoperazine

The activation processes were fitted to a single exponential function to test the kinetics of Kv channel inhibition by trifluoperazine. The time constants for activating the Kv current in the control condition and with 3 μM trifluoperazine were 14.04 ± 0.18 ms

(n = 4) and 13.48 ± 1.19 ms (n = 4), respectively, suggesting that Kv channel activation kinetics were not altered by trifluoperazine.

However, decay of the Kv channel (inactivation process) was accelerated in a trifluoperazine concentration-dependent manner (Fig. 2A). Control Kv currents were slowly and partially decayed due to their intrinsic inactivation [12]. Therefore, in the presence of trifluoperazine, we fitted the data to a double exponential function to obtain two independent time constants using Origin 7.0 software. We assumed that the time constant of the fast component represented the drug-induced blockade of the Kv current (τ_D), and that the slow time constant reflected intrinsic inactivation, similar to previous studies [13,14]. As shown in Fig. 2C, the reciprocal of the time constant (τ_D) for inhibiting the Kv channel at +60 mV was plotted against various trifluoperazine concentrations to determine the apparent association (k_{+1}) and dissociation (k_{-1}) rate constants. According to the fit, we obtained an apparent k_{+1} of 3.73 ± 0.33 $\mu M^{-1}\,s^{-1}$ and an apparent k_{-1} of 5.84 ± 1.41 s^{-1}, respectively, and elicited a theoretical K_d value based on the first-order reaction between the drug and channel ($K_d = k_{-1}/k_{+1}$) of 1.57 μM. This value corresponded closely with the IC_{50} value obtained from the concentration–response curves (1.58 μM, Fig. 2B).

3.4. Effects of trifluoperazine on steady-state activation and inactivation of Kv channels

The steady-state activation and inactivation kinetics of the Kv channel were measured to determine whether the Kv current was inhibited due to a shift in the activation and/or inactivation curves. The activation curve was elicited from the tail current using the typical two-pulse protocol and was fitted with the Boltzmann function. As shown in Fig. 3A, applying 3 μM trifluoperazine induced a significant right shift in the activation curve. The half-maximum activation was shifted by +6 mV in the presence of 3 μM trifluoperazine without a change in the slope (Fig. 3A). In the absence and presence of trifluoperazine, the half-maximum activation potential ($V_{1/2}$) and slope values (k) were −11.87 ± 0.69 mV and 9.08 ± 0.44, respectively, and −5.78 ± 1.05 mV and 8.61 ± 0.46, respectively.

In addition, the steady-state inactivation kinetics of the Kv currents under control conditions and in the presence of trifluoperazine were calculated by fitting with the other Boltzmann equation. As shown in Fig. 3B, 3 μM trifluoperazine did not affect the steady-state inactivation curve. The potential value of half-inactivation ($V_{1/2}$) and the slope value (k) were −32.30 ± 0.25 mV and 6.69 ± 0.22, respectively, for the control and −32.70 ± 0.69 mV and 7.00 ± 0.28, respectively, for 3 μM trifluoperazine. These datas suggest that trifluoperazine changed the voltage sensitivity of the Kv channel, suggesting that trifluoperazine interacts with the Kv channel in the closed state.

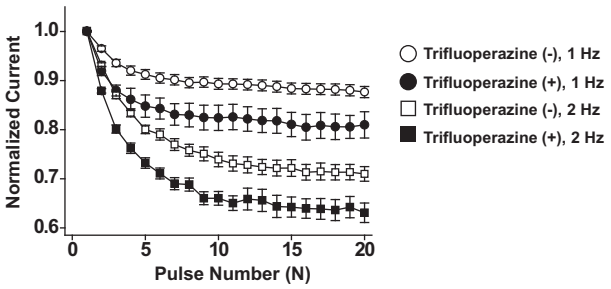

Fig. 4. Use-dependency of blockade of Kv currents by trifluoperazine. In the absence (○, □) and presence of 3 μM trifluoperazine (●, ■), 20 repeated 150-ms depolarizing pulses from holding a potential of −60 mV were applied at frequencies of 1 and 2 Hz, respectively. The normalized current at each frequency was obtained by the peak current amplitude of the first pulse Kv current and plotted vs. the number of pulses n = 6.

3.5. Effects of trifluoperazine on use-dependent inhibition of Kv current

To evaluate the use-dependent inhibition of the Kv channel by trifluoperazine, we applied 20 repeated depolarizing pulses to +60 mV at frequencies of 1 or 2 Hz. In the absence of trifluoperazine, the peak amplitude of the Kv currents decreased by 12% and 29% at 1 and 2 Hz, respectively. In the presence of 3 μM trifluoperazine, the peak amplitudes decreased by 19% and 37% at 1 and 2 Hz, respectively (Fig. 4). These results strongly suggest that trifluoperazine-induced inhibition of the Kv current is frequency (use)-dependent.

4. Discussion

We demonstrated the inhibitory effect of trifluoperazine on the Kv channel in rabbit coronary arterial smooth muscle cells. Our results clearly indicate that trifluoperazine inhibited Kv channels in a state-, time-, and use-dependent manners. Additionally, steady-state inactivation curves of the Kv channels were not affected by trifluoperazine, but steady-state activation curves were shifted toward a more positive potential.

Several lines of evidence support the notion that trifluoperazine inhibits the Kv channel in both open and closed-channel states. Trifluoperazine did not affect the activation time course, whereas the inactivation time course of the Kv current was accelerated in a dose-dependent manner. Therefore, trifluoperazine had a greater

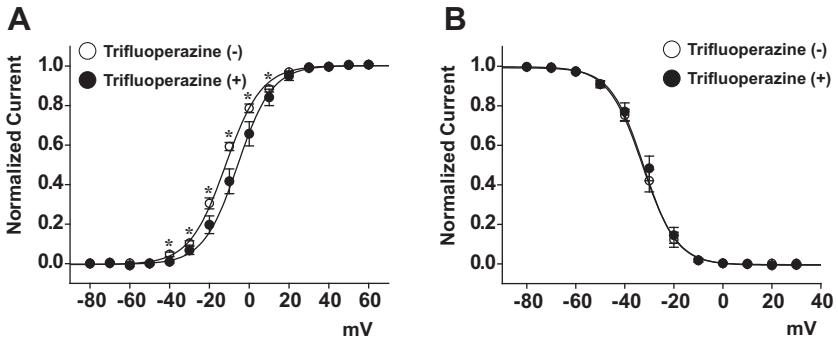

Fig. 3. Influence of trifluoperazine on steady-state activation and inactivation of Kv channels. (A) Activation curves recorded in the absence (○) and presence of 3 μM trifluoperazine (●). To obtain the activation curve data, we used a double-pulse protocol with short (30–100 ms) depolarizing step pulses between −80 and +60 mV at increments of 10 mV and a subsequent return pulse of −40 mV. Current data were normalized from the peak value of the second pulse (tail current), which was used for channel activation. n = 6. $^*P < 0.05$. (B) Steady-state inactivation curves in the absence (○) and presence of 3 μM trifluoperazine (●). The inactivation curve data were elicited by a test step to +40 mV after 7-s conditioning pre-pulses at various voltages. Steady-state current amplitude was normalized to the peak amplitude of pre-pulse potential for the test pulse. These data were fitted to the Boltzmann equation. n = 8.

effect on the steady-state Kv current than on the peak current at the onset of depolarizing pulses (Figs. 1 and 2), suggesting that trifluoperazine blocks Kv channels in the open-state in native coronary arterial smooth muscle cells. Furthermore, blocking the Kv current with trifluoperazine was use-dependent (Fig. 4). These results are commonly observed during open-state blockade [14–16]. Thus, we suggest that the inhibitory effect of trifluoperazine occurred in an open-channel state. However, we also suggest that trifluoperazine interacted with the Kv channels in a closed state. Trifluoperazine shifted the voltage-dependence of the activation curve to a more positive potential without affecting the voltage-dependent inactivation curve (Fig. 3), suggesting that trifluoperazine interacts with the Kv channel in the closed state. Although we could not address the detailed inhibitory mechanism, it should be a direct action regradless of calmodulin, since a calmodulin inhibitor did not alter the effect and the inhibition progressed rapidly, reaching a steady state witin 2 min.

Although clinical use of trifluoperazine has decreased steadily, this drug has been widely used to inhibit calmodulin activity in many laboratories. Calmodulin has crucial roles in many cellular functions such as inflammation, apoptosis, metabolism, immune response, and short- and long-term memory [1]. Dysfunction of calmodulin is closely associated with Alzheimer's disease, Parkinson's disease, and Huntington's disease [5,17,18]. Thus, trifluoperazine is indispensable for studies of the calmodulin-related signaling pathway. However, its usefulness is limited by side effects on other targets including Kv channels. For example, trifluoperazine treatment inhibits the voltage-dependent Kv1.3 channels expressed in human T lymphocytes in a voltage- and time-dependent fashion [19]. Trifluoperazine also inhibits hKv2.1 channels transfected into a human glioblastoma cell line [20], and human *ether-a-go-go*-related gene (HERG) channels expressed in *Xenopus* oocytes [21]. These studies were performed using mainly cultured cell lines; however, no studies have addressed the effect of trifluoperazine on Kv channels in native vascular smooth muscle cells to date. Kv channels play a key role in regulating the resting membrane potential in some vascular smooth muscle, and contribute to resting tone [6,7]. Furthermore, vascular Kv channels are closely related to agonist-induced signaling pathways and vascular contractility. Considering the physiological relevance of Kv channels in vascular smooth muscle and the cellular function of calmodulin, our result suggest caution when using trifluoperazine to study calmodulin-related vascular function.

Most of the side effects on native vascular Kv channels of various chemicals have been identified by our group. For example, LY294002 (a PI3 kinase inhibitor), curcumin (spice), mibefradil (a T-type Ca^{2+} channel inhibitor), verapamil (an L-type Ca^{2+} channel inhibitor), YC-1 (a guanylyl cyclase activator), and bisindolylmaleimide (I) (a PKC inhibitor) directly inhibit the vascular Kv channel in the open state [12–14,16,22,23]. Furthermore, our group also revealed that staurosporine inhibits the vascular Kv channel in both open- and closed-states, similar to the present results [24]. Although the detailed binding mechanisms were not addressed in most studies, these studies provide additional information on the side effects of various chemicals; therefore, care should be taken when using these chemicals as well as trifluoperazine.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.115.

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