Arylbenzazepines Are Potent Modulators for the Delayed Rectifier K$^+$ Channel: A Potential Mechanism for Their Neuroprotective Effects

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Abstract

(±) SKF83959, like many other arylbenzazepines, elicits powerful neuroprotection *in vitro* and *in vivo*. The neuroprotective action of the compound was found to partially depend on its D$_1$-like dopamine receptor agonistic activity. The precise mechanism for the (±) SKF83959-mediated neuroprotection remains elusive. We report here that (±) SKF83959 is a potent blocker for delayed rectifier K$^+$ channel. (±) SKF83959 inhibited the delayed rectifier K$^+$ current (I_K) dose-dependently in rat hippocampal neurons. The IC_{50} value for inhibition of I_K was 41.9±2.3 µM (Hill coefficient = 1.81±0.13, n = 6), whereas that for inhibition of I_A was 307.9±38.5 µM (Hill coefficient = 1.37±0.08, n = 6). Thus, (±) SKF83959 is 7.3-fold more potent in suppressing I_K than I_A. Moreover, the inhibition of I_K by (±) SKF83959 was voltage-dependent and not related to dopamine receptors. The rapidly onset of inhibition and recovery suggests that the inhibition resulted from a direct interaction of (±) SKF83959 with the K$^+$ channel. The intracellular application of (±) SKF83959 had no effects of on I_K, indicating that the compound most likely acts at the outer mouth of the pore of K$^+$ channel. We also tested the enantiomers of (±) SKF83959, R-(+) SKF83959 (MCL-201), and S-(−) SKF83959 (MCL-202), as well as SKF38393; all these compounds inhibited I_K. However, (±) SKF83959, at either 0.1 or 1 mM, exhibited the strongest inhibition on the currents among all tested drug. The present findings not only revealed a new potent blocker of I_K , but also provided a novel mechanism for the neuroprotective action of arylbenzazepines such as (±) SKF83959.

Introduction

Atypical D$_1$ receptor agonist (±) SKF83959, (±) 3-methyl-6-chloro-7,8-hydroxy-1-(3-methylphenyl)-2,3,4,5-tetrahydro-1H-3-benzazepine (Fig. 1), possesses a unique pharmacological property. (±) SKF83959 does not stimulate cAMP formation in brain tissues but activates the phosphatidylinositol (PI)-linked pathway via a D$_1$-like dopamine receptors (D$_1$DAR) [1–5], although it produces D$_1$ agonist-mediated behavioral responses in animals [6]. This compound was shown to have anti-parkinsonian effect in an experimental primate model as well as in the unilateral lesioned rodent model for Parkinson's disease [7–11]. Earlier studies with (±) SKF83959 and its isomers, showed that such D$_1$-like agonists increase eye blinking in monkeys [12] and rats [13] and that the magnitude of this effect may be related to agonistic efficacy. Although there is a recent report by employing a different approach depicting that (±) SKF83959 is able to stimulate native brain tissues coupling to both Gs and Gq protein[14], the antiparkinsonian mechanism of the agent, however, is known not to relate to the cAMP pathway and may associate with the (±) SKF83959-mediated PI-linked D$_1$DAR activation [3,6,15–17]. In

agreement with a previous report in a model of primate Parkinson's disease, in which (±) SKF83959 not only reduced the dyskinesias but also reduced the occurrence of motor fluctuation and wearing-off dyskinesia [15], we have recently demonstrated that chronic treatment of (±) SKF83959 resulted in a significantly lower dyskinesia while eliciting its potent anti-parkinsonian action in a model of 6-hydroxydopamine (6-OHDA) unilaterally -lesioned rats. Moreover, a chronic administration of (±) SKF83959 also significantly reduced levo-DOPA-induced dyskinesias in PD rats [18]. The underlying mechanism for the anti-dyskinesia action of (±) SKF83959 is believed to associate with the drug's powerful neuroprotective action [19]. Since it was suggested that occurrence of motor fluctuation and wearing-off dyskinesia are associated with anti-PD drug treatment-induced further loss of dopaminergic neurons [20,21]. We recently demonstrated that in both cultured neurons and in HEK293 cells, (±) SKF83959 inhibited GSK3β activity via a D$_1$-like receptor-dependent mechanism. However, blockage of D$_1$-like receptor activation which blunted SKF83959-mediated inhibition on GSK3β,was found to attenuated partially the neuroprotective
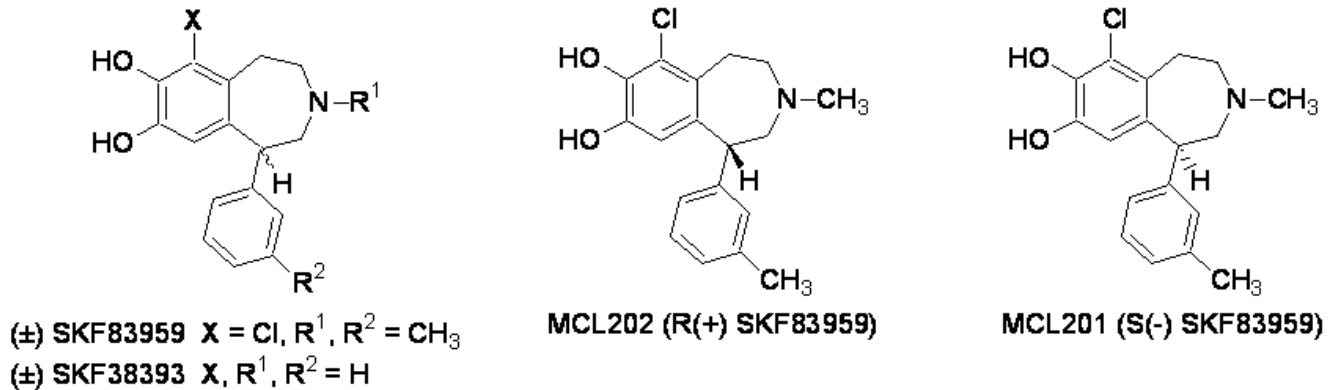
The structure of benzazepines

(±) SKF83959 X = Cl, R^1, R^2 = CH$_3$
(±) SKF38393 X, R^1, R^2 = H

MCL202 (R(+) SKF83959)

MCL201 (S(-) SKF83959)

Figure 1. The structures of benzazepines.
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effect of (±) SKF83959 [19], indicating that D_1-like receptor-independent mechanism may be also involved in the neuroprotection of the drug.

Delayed rectifier K^+ channel plays an important role in neuroprotection [22–24]. It has been proposed that decreased intracellular K^+ due to activation of the K^+ channel contributes to the neuroapoptosis induced by various insults such as Aβ-amyloid [22,25] and serum deprivation [23]. Tetraethylammonium (TEA), a classical blocker of delayed rectifier K^+ channel, was found to exert potent neuroprotective effects *in vivo* [26,27] and *in vitro* [23]. Considering the importance of delayed rectifier K^+ channel in neuroprotection, we decided to explore the potential effect of (±) SKF83959 on delayed rectifier K^+ channel as part of our efforts in understanding the mechanism for the D_1-receptor independent neuroprotection of the drug. We here demonstrate that (±) SKF83959 and related benzazepines are potent inhibitors of delayed rectifier K^+ channel. The potency of (±) SKF83959 is almost 25-fold higher than that of TEA. Our findings not only revealed a new category of potent blockers of delayed rectifier K^+ channel but also provided a novel mechanism for the neuroprotective action of arylylbenzazepines analogs.

Materials and Methods

Materials

(±) SKF38393, (±)-SCH23390 hydrochloride, prazosin and mesulergine were purchased from Sigma (St Louis, MO, USA)/ RBI (Natick, MA, USA). R-(+) SKF38393 (MCL-201) and S-(−) SKF38393 (MCL-202) (Fig. 1) were synthesized in the Alcohol and Drug Abuse Research Center at McLean Hospital (Belmont, MA, USA). (±) SKF83959 was synthesized in the Synthetic Organic & Medicinal Chemistry Laboratory, Shanghai Institute of Materia Medica, Chinese Academy of Sciences. Other chemicals were purchased from Sigma-Aldrich China Inc.

Preparation of dissociated hippocampal neurons

All procedures were in compliance with the Guidelines for the Care and Use of Laboratory Animals (National Research Council, People's Republic of China, 1996). Dissociated hippocampal neurons were prepared from newborn (5–9 day) Sprague-Dawley rats as described previously [26]. Briefly, hippocampal slices (500 μm) were cut in oxygenated ice-cold dissociation solution

consisted of the following (in mM): 82 Na_2SO_4, 30 K_2SO_4, 5 $MgCl_2$, 10 HEPES, and 10 glucose, pH = 7.3 adjusted with NaOH. The slices were incubated in the dissociation solution containing protease XXIII (3 g/L) at 32°C for 8 min, and then placed in dissociation solution containing trypsin inhibitor type II-S (1 g/L) and bovine serum albumin (1 g/L) at 24–25°C under an oxygen atmosphere. Before recording, the CA1 region of several slices was dissected, and triturated using a series of fire-polished Pasteur pipettes with decreasing tip diameters. Dissociated neurons were placed in a recording dish and superfused with an external solution consisted of the following (in mM): 135 NaCl, 5 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 glucose, 0.001 tetrodotoxin , pH = 7.4 adjusted with NaOH..

Whole-cell voltage clamp recoding

Voltage-activated K^+ currents were recorded in large pyramidal-shaped neurons using an Axopatch 200A amplifier (Axon Instruments, USA) at 24–25°C [26]. Voltage protocols were controlled by pClamp 9.0 software via a DigiData-1322A interface (Axon Instruments, USA). Electrodes (a tip resistance of 3–5 MΩ) were pulled from borosilicate grass pipettes (Sutter Instruments, USA) and filled with a pipette solution consisted of the following (in mM): 140 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 10 EGTA (pH 7.4 with KOH). The neurons were held at −50 mV. Unless otherwise mentioned, the total K^+ current (I_{total}) was elicited with 400-ms depolarizing steps to +40 mV following a 600-ms hyperpolarizing prepulse to −110 mV, delivered every 10 s. The delayed rectifier K^+ current (I_K) was elicited by using a similar protocol, but a 50-ms interval at −50 mV was inserted after the prepulse to inactivate the fast transient K^+ current (I_A). I_A is the subtraction of I_K from I_{total}. Signals were filtered at 2–10 KHz and sampled at frequencies of 10–40 KHz. Series resistance was compensated by 75%–85%. Linear leak and residual capacitance currents were subtracted online using a P/4 protocol.

Drug application

(±) SKF83959 and other drugs were dissolved in dimethylsulfoxide (DMSO) to prepare a stock solution of 10 mM and stored at −20°C. Before use, the stock solutions were diluted to desired concentrations. For extracellular application, the drug-containing external solution was delivered to the neuron using RSC-100 rapid solution changer with a 9-tube head (BioLogic Co., France).

DMSO (less than 0.1% in the final dilution) had no observed effect on the voltage-activated K^+ currents (DMSO: 0.598 ± 0.11 nA; control: 0.660 ± 0.095 nA, n = 4; p > 0.05). For intracellular dialysis, ($\pm$) SKF83959 contained in the pipette solution was diffused into the recorded neuron immediately after patch membrane ruptured [29].

Data analysis

The peak amplitude of I_A was measured, whereas the steady-state amplitude of I_K was measured at 300 ms after the initiation of each voltage step. The decay time constants (τ) of the currents were obtained by fitting the decay time course with a mono-exponential function. The concentrations of ($\pm$) SKF83959 to yield 50% block of the K^+ currents (IC_{50}) were obtained by fitting normalized concentration–inhibition relationships to the equation: $I/I_0 = 1/\{1+([C]/IC_{50})^n\}$, where I_0 and I are the current amplitudes measured in control and in the presence of ($\pm$) SKF83959, [C] is the concentration of ($\pm$) SKF83959 in the external solution and n is the Hill coefficient. The ratio of inhibition was calculated by using the equation: Inhibition = $(1-I/I_0)*100\%$, where I_0 and I are the current amplitude in control and in the presence of ($\pm$) SKF83959, respectively. For analyzing the voltage-dependence of steady-state activation or inactivation of I_K, normalized conductance or current was plotted against the membrane potential, and fitted to the Boltzmann equation: $Y = 1/\{1+\exp[(V-V_{1/2})/k]\}$, where Y is the normalized conductance or current, V is the test potential; $V_{1/2}$ is the voltage at half-maximal activation or inactivation of I_K; k is the slope factor. The time course of recovery of I_K from inactivation was fitted with a mono-exponential function: $I/I_{max} = A*\{1-\exp[-\Delta t/\tau]\}$, where I_{max} is the maximal current amplitude; I is the current after a recovery period of Δt; τ is the time constant; A is the amplitude coefficient. Data are presented as mean$\pm$S.E.M. Statistical significance was assessed using a Student's t-test except for the voltage dependence of inhibition of I_K by ($\pm$) SKF83959 where one-way ANOVA test was used, and $P < 0.05$ was considered significant. All analyses were performed using the software "GraphPad Prism 4" and "Excel 2000".

Results

Inhibition of voltage-activated K^+ currents by ($\pm$) SKF83959 in hippoacampal neurons

Superfusion with ($\pm$) SKF83959 (10–1000 µM) inhibited both I_K and I_A in concentration-dependent manner. However, the compound preferentially inhibited I_K to I_A. As shown in Fig. 2A, ($\pm$) SKF83959 (100 µM) markedly suppressed I_K, whereas a moderate reduction in the amplitude of I_A was observed. The inhibition of ($\pm$) SKF83959 on both K^+ currents had a rapid onset, as it reached steady-state levels within 10 s. Moreover, the K^+ currents were partially recovered upon washing out the compound (Fig. 2B, 2C). The partial recovery may be because of the run-down of the currents with recording, or due to the incompletely drug washing- out.

Analyzing the concentration-inhibition relationships of ($\pm$) SKF83959 on K^+ currents revealed that the IC_{50} value for inhibition of I_K was 41.9 ± 2.3 µM (Hill coefficient = 1.81 ± 0.13, n = 6), while that for inhibition of I_A was 307.9 ± 38.5 µM (Hill coefficient = 1.37 ± 0.08, n = 6) (Fig. 3A). Because ($\pm$) SKF83959 is 7.3-fold more potent in suppressing I_K than I_A, we thus focused on characterizing the inhibition on I_K.

We next examined whether the other substituted phenylbenzazepines such as R-(+) SKF83959 (MCL-201), S-(−) SKF83959 (MCL-202) and SKF38393 affect I_K. The results are shown in Fig. 3B. At the concentration of 100 µM, the inhibition of I_K by MCL-201, MCL-202 and SKF38393 was $36.7\pm4.1\%$ (n = 8), $9.7\pm1.7\%$ (n = 8) and $24.5\pm1.1\%$ (n = 7), respectively, whereas ($\pm$) SKF83959 (100 µM) induced much greater inhibition on I_K ($76.0\pm2.7\%$, n = 6). At the concentration of 1 mM, the inhibition of I_K by MCL-201, MCL-202 and SKF38393 was $77.1\pm4.0\%$ (n = 3), $56.1\pm2.7\%$ (n = 4) and $62.0\pm2.7\%$ (n = 5), respectively, whereas ($\pm$) SKF83959 (1 mM) almost completely suppress the K^+ current ($92.0\pm2.5\%$, n = 3). Thus, ($\pm$) SKF83959 produced the most potent inhibition of I_K among the four substituted benzazepines at tested concentrations.

Mechanisms underlying the inhibition of the delayed rectifier K^+ current by ($\pm$) SKF83959

($\pm$) SKF83959 is an atypical agonist of D_1-like receptor. We first examined whether the inhibition of I_K by ($\pm$) SKF83959 was mediated through activation of D_1-like receptor. Pretreatment with the D_1 receptor antagonist SCH23390 did not significantly alter ($\pm$) SKF83959-induced inhibition on I_K (Fig. 4A). The inhibition of I_K by ($\pm$) SKF83959 (100 µM) in the presence of SCH23390 (20 µM) was $60.1\pm2.7\%$ (n = 6), while that by ($\pm\pm$) SKF83959 alone was $70.3\pm4.1\%$ (n = 7, $P = 0.074$, t = 1.97, df = 11).

In addition to D_1-like receptor, ($\pm$) SKF83959 also exhibits moderate affinities to D_2 receptors, α_1-adrenoceptors and $5-HT_{2A}$ receptors [5]. However, neither the α1-adrenoceptors antagonist prazosin, nor the 5-HT receptor antagonist mesulergine affected ($\pm$) SKF83959-induced inhibition of I_K (Fig. 4A). The inhibition of I_K by ($\pm$) SKF83959 (100 µM) in the presence of prazosin (10 µM) or mesulergine (10 µM) was $76.6\pm2.1\%$ (n = 6) and $71.4\pm4.6\%$ (n = 6), respectively, which was almost identical with that of ($\pm$) SKF83959 alone treatment ($70.3\pm4.1\%$, n = 7, $P = 0.23$, t = 1.29, df = 11 vs. prazosin and $P = 0.86$, t = 0.18, df = 11 vs. mesulergine). Taken together, ($\pm$) SKF83959 is a potent blocker of the delayed rectifier K^+ channel and the inhibitory action is independent of either dopamine or serotonin receptors.

To determine the acting site of the agent on the K^+ channel, we investigated the effect of intracellular dialysis of ($\pm$) SKF83959 on I_K. The concentration for intracellular dialysis was 300 µM, which inhibited I_K by nearly 90% and is close to the value of IC50 for I_A, when applied externally (Fig. 3A). Throughout the 10-min recording period, the relative amplitudes of I_K in the neurons dialyzed with ($\pm$) SKF83959 were almost identical with that of in the control group (Fig. 4B), so was the I_K (data not shown). At the end of 10-min recording, the relative amplitude of I_K in control and in the neurons dialyzed with ($\pm$) SKF83959 was $85.4\pm6.0\%$ (n = 5) and $83.4\pm5.3\%$ (n = 7), respectively ($P = 0.81$, t = 0.25, df = 10). The results suggest that ($\pm$) SKF83959 acts at an extracellular site of the delayed rectifier K^+ channel.

Voltage dependence of inhibition of the delayed rectifier K^+ current by ($\pm$) SKF83959

The current-voltage (I/V) relationship of I_K from a representative neuron in control and in the presence of ($\pm$) SKF83959 (100 µM) was plotted in Fig. 5A. ($\pm$) SKF83959 did not significantly change the threshold for activation of I_K, but caused a remarkable downward shift of the I/V curve, and reduced its amplitude over the entire period of activation. The inhibition of I_K by ($\pm$) SKF83959 seems to be greater at more depolarizing potentials. Thus, we plotted the relative amplitudes of I_K as a function of the test potential. As shown in Fig. 5B, the inhibition of I_K by ($\pm$) SKF83959 steeply increased between −20 mV and +20 mV. In the presence of ($\pm$) SKF83959, the relative currents in steps to −20 mV and to +20 mV, respectively, were $43.5\pm6.1\%$
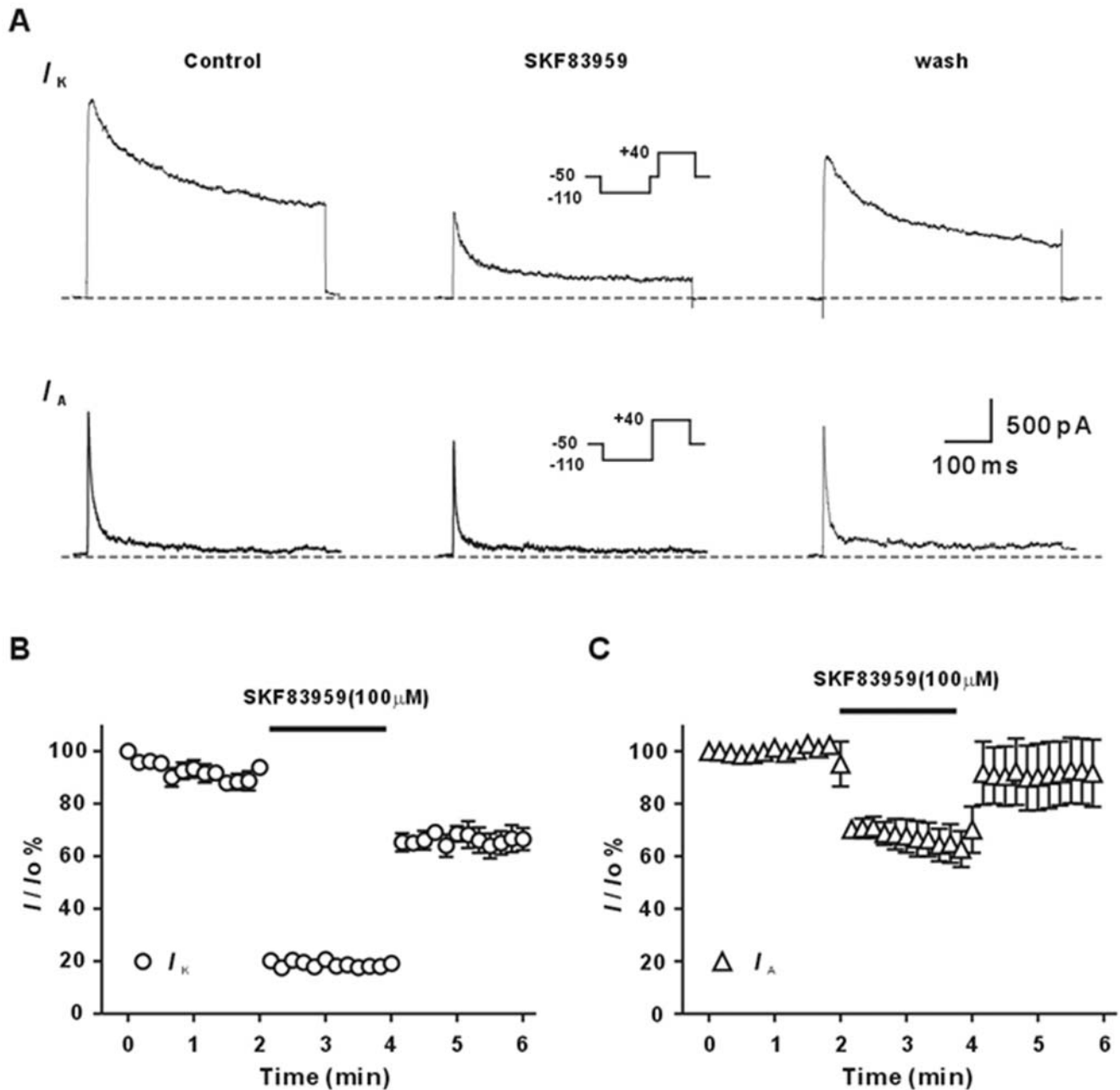
Figure 2. Inhibition of voltage-activated K$^+$ currents by ($\pm$) SKF83959 in rat hippocampal neurons. (A) Upper and lower are the respective representative traces of the delayed rectifier K$^+$ current (I_K) and fast transient K$^+$ current (I_A) recorded prior to and during superfusion with ($\pm$) SKF83959 (100 µM) and after 10 s of washout. The neuron was held at -50 mV. Upper inset shows the pulse protocol to elicit I_K, whereas lower inset shows the protocol to elicit the total K$^+$ current. I_A is the subtraction of I_K from the total K$^+$ current. (B) and (C) Time courses of the inhibition of I_K and I_A by ($\pm$) SKF83959 (100 µM, n = 5 for each). The bar denotes the surpufusion with SKF83959. A number of symbols in (B) and (C) have error bars smaller than their size.
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and $18.1\pm1.8\%$ (n = 7, $P = 0.0019$, t = 3.96, df = 12). Between +20 mV and +80 mV the inhibition remained relatively constant. The results demonstrated that the inhibition of I_K by ($\pm$) SKF83959 was voltage-dependent.

Effects of ($\pm$) SKF83959 on kinetic behaviors of the delayed rectifier K$^+$ current

In addition to suppressing the amplitude of I_K, ($\pm$) SKF83959 (100 µM) markedly accelerated the decay of the current (Fig. 6A&6B). The time course of the decay of the current trace was fitted with a single exponential function. The decay time constant (τ) for I_K was 145.9 ± 11.9 ms in control and 63.4 ± 4.9 ms in the presence of 100 µM ($\pm$) SKF83959 (n = 7, $P = 0.0002$, t = 6.42, df = 12, vs. control) (Fig. 6C). Moreover, as depicted in Fig. 6D, ($\pm$) SKF83959 suppressed the amplitude of tail current, but did not cause a crossover of the tail current.

($\pm$) SKF83959 (100 µM) elicited a marked hyperpolarizing shift (nearly 20 mV) of the voltage dependence of steady-state
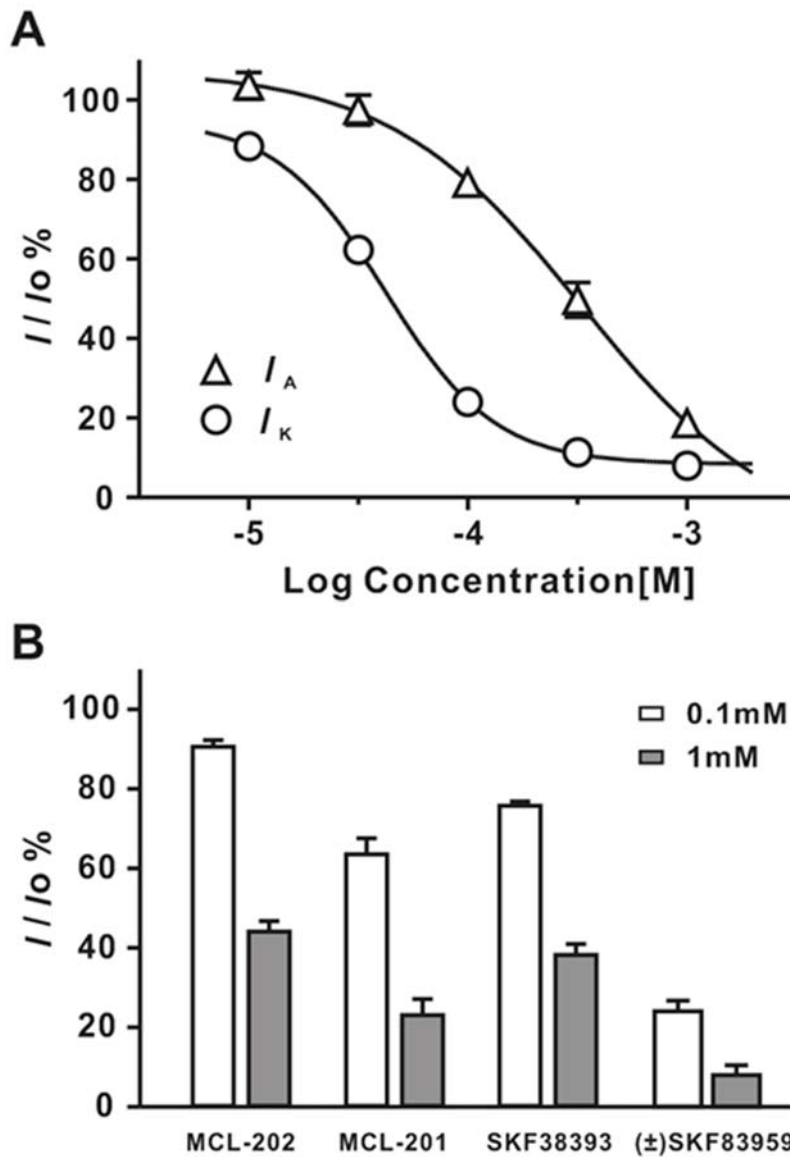
Figure 3. Concentration-dependent inhibition of (±) SKF83959 on the voltage-activated K$^+$ currents. (A) Concentration-inhibition curves of (±) SKF83959 on the delayed rectifier K$^+$ current (I_K) and fast transient K$^+$ current (I_A)(n = 6 for I_K and I_A). A number of symbols have error bars smaller than their size. (B) Comparison of the substituted phenylbenzazepine, MCL-201, MCL-202, SKF38393 and (±) SKF83959 in inhibition of I_K.
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activation curve of I_K (Fig. 7A). The value of $V_{1/2}$ for activation was changed from -1.7 ± 2.6 mV to -22.2 ± 3.1 mV (n = 8, $P = 0.0002$, t = 5.10, df = 14), whereas the value of k for activation was from 16.8 ± 0.9 to 13.6 ± 1.1, n = 8, $P = 0.038$, t = 2.29, df = 14). (±) SKF83959 (100 µM) had no significant effect on its steady-state inactivation (Fig. 7B). The value of $V_{1/2}$ for inactivation was nearly identical (-85.8 ± 1.3 mV in control $vs. -86.3\pm3.9$ mV in the presence of (±) SKF83959, n = 8, $P = 0.91$, t = 0.11, df = 12). The value of k for inactivation has no significant change (-11.9 ± 0.7 in control $vs. -16.7\pm4.8$ in the presence of (±) SKF83959, n = 8, $P = 0.35$, t = 1.00, df = 6). The same treatment accelerated the recovery of I_K from inactivation (Fig. 7C). The time constant of recovery was found to reduce from 294.1 ± 30.4 ms to 107.7 ± 10.4 ms (n = 6, $P = 0.0002$, t = 5.81, df = 10).

Discussion

(±) SKF83959 is a putative phosphatidylinositol (PI)-linked D$_1$-like receptor agonist [3,4]. The agent has been shown to possess potent anti-parkinsonian effects in a variety of animal models for Parkinson's disease with less severe dyskinesia and motor fluctuation [6–8,15,18]. In the present study, we demonstrated for the first time that (±) SKF83959 and other substituted phenylbenzazepines inhibited the delayed rectifier K$^+$ current (I_K) in rat hippocampal neurons with the highest inhibition produced by (±) SKF83959. (±) SKF83959 is nearly 25-fold more potent than tetraethylammonium (TEA), a classical blocker of I_K, which had an IC_{50} value of 1.05 ± 0.21 mM in the same preparations [28].

To elucidate the mechanisms underlying (±) SKF83959-induced inhibition on I_K, we found that the antagonists of D$_1$,
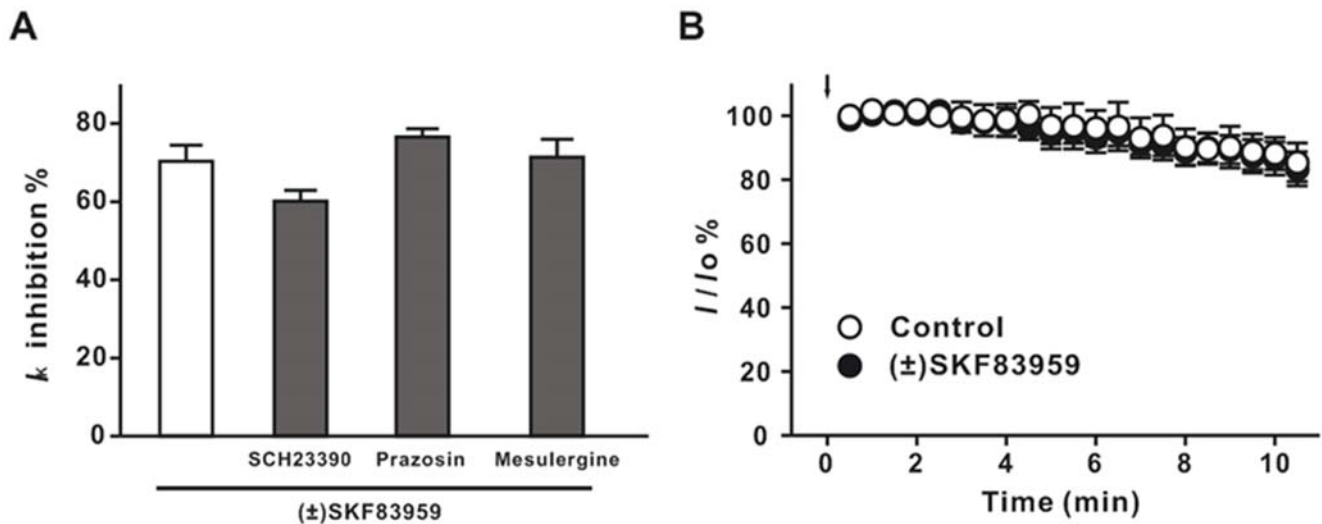
Figure 4. Block of the delayed rectifier K$^+$ channel by ($\pm$) SKF83959 in rat hippocampal neurons. (A) Inhibition of the delayed rectifier K$^+$ current (I_K) by ($\pm$) SKF83959 (100 μM) in the presence of D$_1$ receptor antagonist SCH23390 (20 μM, n = 6), or α1-adrenoceptor antagonist prazosin (10 μM, n = 6), or 5-HT receptor antagonist mesulergine (10 μM, n = 6). (B) Lacking effect of intracellular dialysis of ($\pm$) SKF83959 on I_K (n = 7). The recording pipettes were filled with pipette solution containing ($\pm$) SKF83959 (300 μM). The downward arrow indicates the time when the patch membrane was ruptured.
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D$_2$ or 5-HT$_{1A}$ receptors did not block the inhibition of I_K by ($\pm$) SKF83959 (Fig. 3A), indicating that the inhibitory effect was a receptor-independent event. Furthermore, the fast nature in the onset of inhibition and recovery (Fig. 2B) implicates that the inhibition results from a direct interaction of ($\pm$) SKF83959 with the K$^+$ channel. Moreover, we found that intracellular application of ($\pm$) SKF83959 had no effect on I_K (Fig. 4B), suggesting that the agent is most likely to act at the outer mouth of the pore of K$^+$ channel. In an effort to determine how ($\pm$) SKF83959 blocks the K$^+$ channel. We demonstrated that the inhibition of I_K by ($\pm$) SKF83959 was voltage-dependent (Fig. 5). Furthermore, the agent markedly accelerated the decay of I_K in addition to suppressing its

amplitude (Fig. 6A), although lack of the crossover of tail current (Fig. 6D). The results suggest that the agent preferentially binds to the open state of the K$^+$ channel [30,31]. It is conceivable that ($\pm$) SKF83959 acts as an open-channel blocker at the delayed rectifier K$^+$ channel. ($\pm$) SKF83959 probably binds to the K$^+$ channel with 2:1 stoichometry (Hill coefficient = 1.81±0.13).

Accumulating evidence shows that loss of intracellular K$^+$ ions through enhanced delayed rectifier K$^+$ channel (mainly Kv2.1 channel) mediates apoptosis of cortical neurons induced by a variety of treatments, such as serum deprivation, exposure to staurosporine or β–amyloid peptide fragment, etc. [23,25]. In 6-hydroxydopamine -induced neurotoxicity, voltage-dependent po-
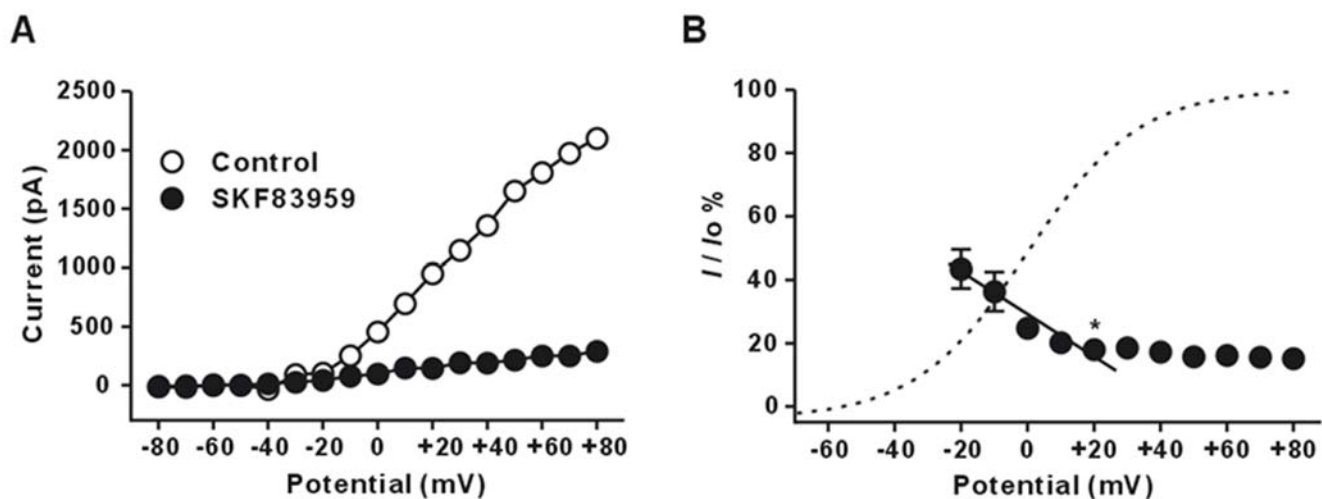

Figure 5. Effects of ($\pm$) SKF83959 on the current-voltage relationships of the delayed rectifier K$^+$ current in rat hippocampal neurons. (A) Current-voltage (I/V) curves of the delayed rectifier K$^+$ current (I_K) obtained in a representative neuron in control and during superfusion of ($\pm$) SKF83959 (100 μM). (B) Plot of the relative amplitudes of I_K during superfusion of ($\pm$) SKF83959 (100 μM) as a function of the test potential. Each symbol represents the mean±S.E.M. (n = 7). The solid line is the linear fit, while the dashed line is the activation curve obtained in control condition. *$P < 0.01$ (one-way ANOVA test) versus the value at −20 mV.
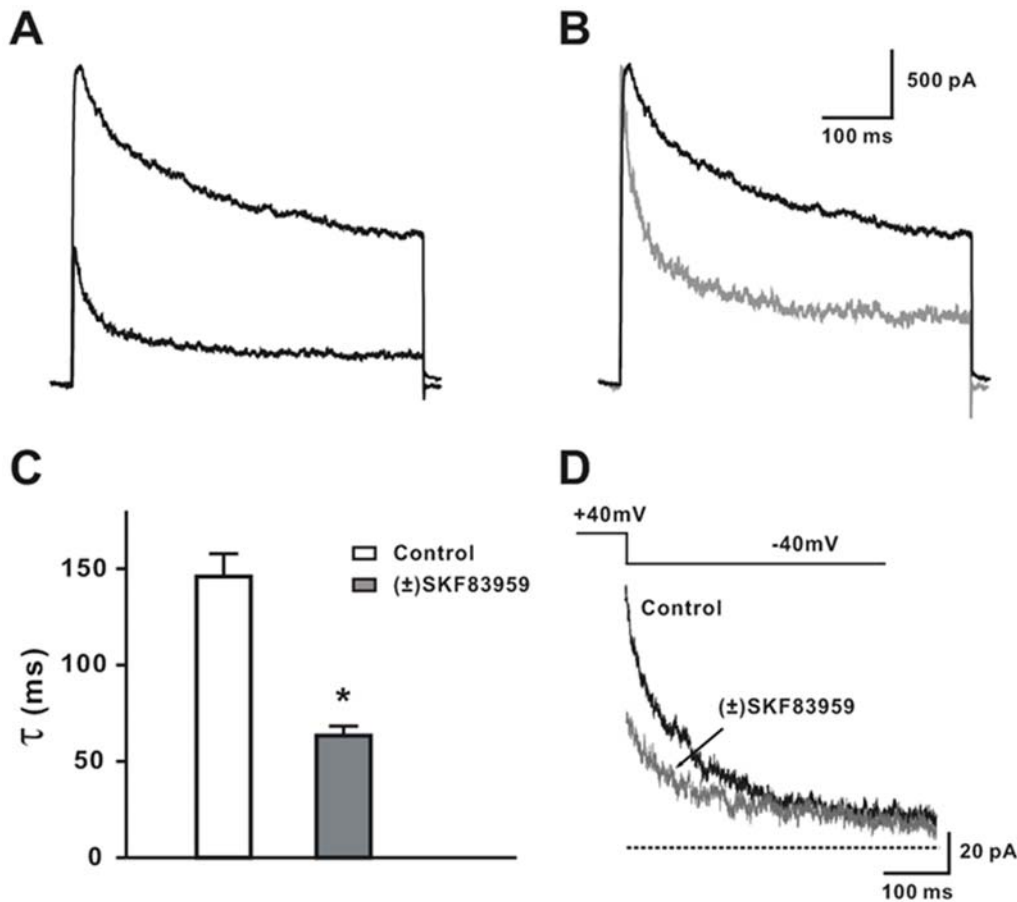doi:10.1371/journal.pone.0005811.g005

Figure 6. Effects of ($\pm$) SKF83959 on the decay of the delayed rectifier K$^+$ current in rat hippocampal neurons. (A) Superimposed traces of the delayed rectifier K$^+$ current (I_K) in a representative neuron prior to and during superfusion with ($\pm$) SKF83959 (100 µM). (B) The trace with ($\pm$) SKF83959 in (A) is scaled up. (C) Decay time constants of I_K prior to and during superfusion with ($\pm$) SKF83959 (100 µM). n = 7, *P<0.01 *versus* the control. (D) Tail current traces evoked at −40 mV after a 500-ms depolarizing steps to +40 mV prior to and during superfusion with ($\pm$) SKF83959 (100 µM). Similar results were obtained in 3 neurons.
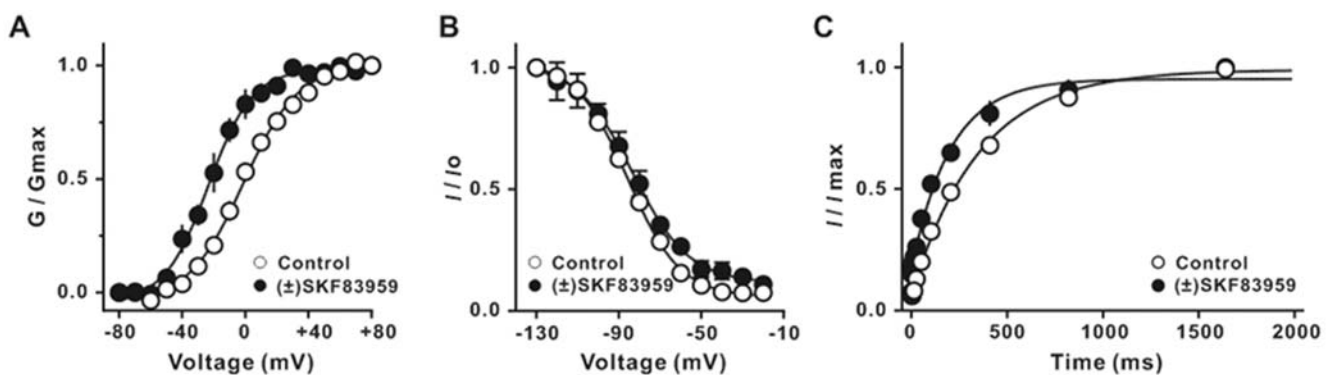doi:10.1371/journal.pone.0005811.g006

Figure 7. Effects of ($\pm$) SKF83959 on the activation and steady-state inactivation of the delayed rectifier K$^+$ current in rat hippocampal neurons. (A) Activation curves of the delayed rectifier K$^+$ current (I_K) prior to and during superfusion with 100 µM ($\pm$) SKF83959 (n = 8). (B) Steady-state inactivation curves of I_K prior to and during superfusion with 100 µM ($\pm$) SKF83959 (n = 8). (C) Time courses of recovery of I_K from inactivation prior to and during superfusion with 100 µM ($\pm$) SKF83959 (n = 6). For studying the activation, neurons were held at −50 mV, currents were elicited with a series of 400-ms step from −80 mV to +80 mV in 10 mV increments following a 600-ms hyperpolarizing prepulse to −110 mV and a 50-ms interval at −50 mV, delivered every 10 s. For studying the steady-state inactivation, neurons were held at 0 mV, currents were elicited with a series of 600-ms prepulses at different hyperpolarizing potentials followed by a 50-ms interval at −50 mV and a 400-ms step to +40 mV, then back to 0 mV, delivered every 10 s. For studying the time course of recovery from inactivation, neurons were held at 0 mV, currents were elicited on return from hyperpolarizing prepulses of varying durations at −110 mV to +40 mV, delivered every 10 s.
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tassium channels were found to play a vital role [32], since blockage of I_K by TEA was shown to effectively against the neurotoxicity and improve the neuronal viability. In other hand, the neuroprotective effects of classical I_K blocker TEA were also demonstrated in animal models of transient focal ischemia [26,27]. Thus, the drug discovery targeted to specific K^+ channel has been proposed as a potential therapeutic approach in treatment of neurodegenerative diseases [33–35]. We recently demonstrated that (±) SKF83959 protected rat cortical neurons against H_2O_2-induced injury, which was partially mediated though a putative PI-linked D_1-like receptor-dependent mechanism via inhibition of GSK3β pathway [19]. The finding of (±) SKF83959 and its analogs as a potent blocker for the delayed rectifier K^+ channel in the present study not only reveals a role of those compounds in the

modulation of K^+ channel, but also suggests a potential new mechanism for the neuroprotection of phenylbenzazepine derivatives. In the case of (±) SKF83959, how the neuroprotective effects via blockage of the delayed rectifier K^+ channel contributes to the observed attenuation in the development of dyskinesia and reduction in the occurrence of motor fluctuation and wearing-off dyskinesia in chronic (±) SKF83959-treated PD animals [7,18] are worth of further study.

Author Contributions

Conceived and designed the experiments: GZJ GYH AZ XZ. Performed the experiments: XC JZ. Analyzed the data: GYH AZ. Contributed reagents/materials/analysis tools: JZ JLN AZ. Wrote the paper: JLN XZ.

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