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 D₁-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 (Iₑ) dose-dependently in rat hippocampal neurons. The IC₅₀ value for inhibition of Iₑ was 41.9 ± 2.3 μM (Hill coefficient = 1.81 ± 0.13, n = 6), whereas that for inhibition of Iₑ was 307.9 ± 38.5 μM (Hill coefficient = 1.37 ± 0.08, n = 6). Thus, (±) SKF83959 is 7.3-fold more potent in suppressing Iₑ than Iₑ. Moreover, the inhibition of Iₑ 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 on Iₑ, 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 SKF83839; all these compounds inhibited Iₑ. 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 Iₑ, but also provided a novel mechanism for the neuroprotective action of arylbenzazepines such as (±) SKF83959.

Introduction

Atypical D₁ 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 D₁-like dopamine receptors (D₁DAR) [1–5], although it produces D₁ 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 D₁-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 D₁DAR 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 levodopa-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 is 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 D₁-like receptor-dependent mechanism. However, blockage of D₁-like receptor activation which blunted SKF83959-mediated inhibition on GSK3β, was found to attenuated partially the neuroprotective...
effect of (±) SKF83959 [19], indicating that D1-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 D₁-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

(±) SKF83893, (±)-SCH23390 hydrochloride, prazosin and mesulergine were purchased from Sigma (St Louis, MO, USA)/RBI (Natick, MA, USA). R(+) SKF83893 (MCL-201) and S(-) SKF83893 (MCL-202) (Fig. 1) were synthesized in the Alcohol and Drug Abuse Research Center at McLean Hospital (Belmont, MA, USA) and filled with a pipette solution consisted of the following (in mM): 140 KCl, 2 MgCl₂, 1 CaCl₂, 10 HEPES, 10 glucose, pH = 7.4 adjusted with NaOH.

Whole-cell voltage clamp recording

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 MgCl₂, 1 CaCl₂, 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_{Kd}) 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_{Ks}). I_{Kd} is the subtraction of I_{Ks} 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).
membrane ruptured [29]. 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 ($\tau$) 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: $\text{Inhibition} = 1 - (I/I_0)*100\%$, 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: $Y = 1/(1+\exp[\theta - V/V_t]/k])$, where $\theta$ is the normalized conductance or current, $V$ is the test potential; $V_t/k$ 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: $I/I_{max} = \Lambda*\exp[-\Delta t/\tau]$, where $I_{max}$ is the maximal current amplitude; $I$ is the current after a recovery period of $\Delta t$; $\tau$ is the time constant; $\Lambda$ is the amplitude coefficient. Data are presented as mean±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 hippocampal 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 = 1.31±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 phenylbenzaazepines such as R- (+) SKF83959 (MCL-201), S- (+) SKF83959 (MCL-202) and SKF83993 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±4.1% (n = 8), 9.7±1.7% (n = 6) and 24.3±1.1% (n = 7), respectively, whereas ($\pm$) SKF83959 (100 μM) induced much greater inhibition on $I_K$ (76.0±2.7%, n = 6). At the concentration of 1 mM, the inhibition of $I_K$ by MCL-201, MCL-202 and SKF38393 was 71.1±4.0% (n = 3), 56.1±2.7% (n = 4) and 62.0±2.7% (n = 5), respectively, whereas ($\pm$) SKF83959 (1 mM) almost completely suppress the $K^+$ current (92.0±2.5%, 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 D1-like receptor. We first examined whether the inhibition of $I_K$ by ($\pm$) SKF83959 was mediated through activation of D1-like receptor. Pretreatment with the D1 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 60.2±2.7% (n = 6), while that by ($\pm$) SKF83959 alone was 70.3±4.1% (n = 7, P = 0.074, t = 1.97, df = 11).

In addition to D1-like receptor, ($\pm$) SKF83959 also exhibits moderate affinities to D2 receptors, a1-adrenoceptors and 5-HT2A receptors [5]. However, neither the a1-adrenoceptors antagonist prazosin, nor the 5-HT receptors 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±2.1% (n = 6) and 71.4±4.6% (n = 6), respectively, which was almost identical with that of ($\pm$) SKF83959 alone treatment (70.3±4.1%, n = 7, P = 0.23, t = 1.29, df = 11 vs. prazosin and $P = 0.06$, t = 1.08, 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 $IC_{50}$ for $I_K$, 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 65.4±6.0% (n = 5) and 63.4±5.3% (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±6.1%
and $18.1 \pm 1.8\%$ ($n = 7$, $P = 0.0019$, $t = 3.96$, 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 ($\tau$) for $I_K$ was $145.9 \pm 11.9$ ms in control and $63.4 \pm 4.9$ 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
activation curve of $I_K$ (Fig. 7A). The value of $V_{1/2}$ for activation was changed from $-1.7 \pm 2.6$ mV to $-22.2 \pm 3.1$ mV ($n=8$, $P=0.0002$, $t=5.10$, df = 14), whereas the value of $k$ for activation was from $16.8 \pm 0.9$ to $13.6 \pm 1.1$, $n=8$, $P=0.038$, $t=2.29$, df = 14). ($\pm$) SKF83959 (100 $\mu$M) had no significant effect on its steady-state inactivation (Fig. 7B). The value of $V_{1/2}$ for inactivation was nearly identical ($-85.8 \pm 1.3$ mV in control vs. $-86.3 \pm 3.9$ mV in the presence of ($\pm$) SKF83959, $n=8$, $P=0.91$, $t=0.11$, df = 12). The value of $k$ for inactivation has no significant change ($-11.9 \pm 0.7$ in control vs. $-16.7 \pm 4.8$ in the presence of ($\pm$) 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 $294.1 \pm 30.4$ ms to $107.7 \pm 10.4$ ms ($n=6$, $P=0.0002$, $t=5.81$, df = 10).

**Discussion**

($\pm$) 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 ($\pm$) SKF83959 and other substituted phenylbenzazepines inhibited the delayed rectifier $K^+$ current ($I_K$) in rat hippocampal neurons with the highest inhibition produced by ($\pm$) SKF83959. ($\pm$) SKF83959 is nearly 25-fold more potent than tetraethylammonium (TEA), a classical blocker of $I_K$, which had an IC$_{50}$ value of $1.05 \pm 0.21$ mM in the same preparations [28].

To elucidate the mechanisms underlying ($\pm$) SKF83959-induced inhibition on $I_K$, we found that the antagonists of D$_1$,
D₂ or 5-HT₁A receptors did not block the inhibition of \( I_K \) by (±) 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 (±) SKF83959 with the \( K^+ \) channel. Moreover, we found that intracellular application of (±) 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 (±) SKF83959 blocks the \( K^+ \) channel, we demonstrated that the inhibition of \( I_K \) by (±) 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 (±) SKF83959 acts as an open-channel blocker at the delayed rectifier \( K^+ \) channel. (±) SKF83959 probably binds to the \( K^+ \) channel with 2:1 stoichiometry (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 6. Effects of (±) 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 (±) SKF83959 (100 μM). (B) The trace with (±) SKF83959 in (A) is scaled up. (C) Decay time constants of I_K prior to and during superfusion with (±) 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 (±) SKF83959 (100 μM). Similar results were obtained in 3 neurons.

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Figure 7. Effects of (±) 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 (±) SKF83959 (n = 8). (B) Steady-state inactivation curves of I_K prior to and during superfusion with 100 μM (±) SKF83959 (n = 8). (C) Time courses of recovery of I_K from inactivation prior to and during superfusion with 100 μM (±) 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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tassion 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 efficacy targeted to apyritic $I_K$ channel has been proposed as a potential therapeutic approach in treatment of neurodegenerative diseases [33–35]. We recently demonstrated that ($\pm$) SKF83959 protected rat cortical neurons against $H_2O_2$-induced injury, which was partially mediated through a putative PI-linked $D_1$-like receptor-dependent mechanism via inhibition of GSK3β pathway [19]. The finding of ($\pm$) 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 ($\pm$) 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 ($\pm$) 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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**References**