

Effect of sodium ferulate on delayed rectifier K^+ currents in PC12 cells

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Abstract. In order to investigate the effect of sodium ferulate (SF) on voltage-activated K^+ channels, the delayed rectifier K^+ current (I_k) in PC12 rat pheochromocytoma cells was recorded using the automated patch-clamp method. The results indicated that following the application of SF, the I_k in PC12 cells was significantly decreased in a concentration-dependent manner. The analysis of activation kinetic curves and inactivation kinetic curves of I_k showed that SF had an effect on the activation and inactivation kinetics. Following the application of 15.3 μ M SF, the activation curve of the I_k of PC12 cells was shifted to positive potentials and the inactivation curve of the I_k of PC12 cells was shifted to negative potentials. This study revealed that the delayed rectifier K^+ currents of PC12 cells were inhibited following SF treatment in a concentration-dependent manner. The mechanism may be associated with the delayed activation and enhanced inactivation of I_k -associated channels.

Introduction

Sodium ferulate (SF), the sodium salt of ferulic acid, a type of organic acid extracted from the traditional Chinese herb *Radix Angelicae Sinensis* (Oliv.) Diels, is an approved drug used for the treatment of cardiovascular and cerebrovascular diseases in China. Pharmacological effects, including anti-oxidative and anti-inflammatory activity, inhibition of platelet aggregation and free radical-scavenging activity have been demonstrated (1,2). It has been reported that SF may decrease the pain transmitted during chronic neuropathic pain injury (3). Therefore, the present study investigated whether SF has an effect on ion channels. Ion channels of various types are associated with neuronal excitability since they are important physiological regulators of action potential shape, membrane potential and firing adaptation in excitable

tissues (4,5). In particular, the delayed rectifier K^+ current (I_k), which is representative of the voltage-gated K^+ (K_v) channel, has been previously investigated in order to determine the anti-nociceptive mechanism (6,7) since it has an important role in cellular signaling processes, including membrane excitability and neurotransmitter release (8,9).

The patch-clamp technique is a state-of-the-art technology for the study of ion channels; however, patch clamping is a laborious process, which requires skilled and trained individuals. Recently, the automated patch-clamp technique has been developed, and research investigating cell channels has increased markedly. It allows for better qualification of hits and shortens the length of time required to study novel drugs (10,11). However, it is difficult to capture primary neurons in order to record the I_k for neuropathic pain studies since there are a large number of glial cells in the original generation of cultured cells, which increases the capture error. Therefore, undifferentiated PC12 cells, chromaffin-like cells derived from rat adrenal medulla that express the I_k and exhibit typical neuronal characteristics in form and function, are widely used as an *in vitro* model for neuron research (12,13).

In the present study, the effect of SF on the I_k through the K_v channel of neuronal PC12 cells was determined using an automated patch-clamp method. The results may provide data useful in explaining the mechanism of the analgesic effect of SF.

Materials and methods

Drugs and chemicals. SF [molecular formula, $C_{10}H_9NaO_4 \cdot 2H_2O$; molecular weight, 252.20; CAS, 24276-84-4; high-performance liquid chromatography purity, >98%) was provided by Beijing SL Pharmaceutical Co., Ltd. (Beijing, China). N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and ethyleneglycol bis(β)-aminoethyl ether N,N,N',N'-tetraacetic acid (EGTA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and heat-inactivated horse serum (HS) were purchased from Gibco (Grand Island, NY, USA). The other reagents were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

Cell culture. Undifferentiated PC12 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in DMEM, supplemented with 5% FBS, 10% HS, 100 U/ml peni-

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cillin, 2 mM glutamine and 100 mg/ml streptomycin, in the absence of a nerve growth factor (14). Cells were maintained on Petri dishes at 37°C, in a 5% CO₂ humidified atmosphere. The culture medium was changed every 3-5 days and cells were split when necessary.

Solutions. For the I_k recording, the composition of the standard external solution was as follows: 5 mmol/l KCl, 145 mmol/l NaCl, 2 mmol/l CaCl₂, 1 mmol/l MgCl₂, 10 mmol/l HEPES and 10 mmol/l D-glucose monohydrate, with the pH adjusted to 7.3 with NaOH. The internal solution was as follows: 1 mmol/l MgCl₂, 135 mmol/l KCl, 1 mmol/l EGTA, 10 mmol/l HEPES, 1 mmol/l ATPNa₂ and 10 mmol/l glucose, with the pH adjusted to 7.3 with KOH. The seal enhancer, which was used to assist stable seal formation at the seal formation step, was as follows: 3 mmol/l KCl, 80 mmol/l NaCl, 35 mmol/l CaCl₂, 10 mmol/l HEPES and 10 mmol/l MgCl₂, with the pH adjusted to 7.2 with NaOH.

Automated patch-clamp current recording. The automated patch-clamp device (NPC-16 Patchliner; Nanion Technologies, Munich, Germany), with a low-pass filter (10 kHz), a 4-pole Bessel filter and EPC 10 Patch Clamp Amplifiers (HEKA Elektronik, Lambrecht/Pfalz, Germany), was used to record the whole-cell I_k. The patch solutions and cells were automatically acquired and they were added to the four wells in the microfabricated disposable chip. All the experiments were performed at room temperature (22-24°C).

In order to record the I_k, the holding potential was set to -70 mV. A 30 msec conditioning depolarization at -40 mV then followed in order to inactivate the Na⁺ channels. 10-mV step pulses (200 msec duration) between -60 and +40 mV were applied. I_k was evoked and did not decay in the 200-msec course. In order to indicate a delayed rectifier K⁺ current, the peak outward currents were blocked to 22.0±1.4% of the control values with 5 mM tetraethylammonium, as previously described for undifferentiated PC12 cells (15). For recording the effect of SF on I_k, four concentration of SF were used: 3.8, 7.7, 15.3 and 30.6 μM. Each concentration of SF was added once to the cells and maintained for ≥300 sec until currents reached equilibrium. In each group, five valid data cells were recorded (n=5). Peak amplitudes of the currents were measured. The normalized current was calculated using the formula: Normalized current = I_{drug}/I × 10 (where I_{drug} and I are peak amplitudes of I_k following and prior to the application of SF, respectively).

In order to record the activation kinetic curves of I_k, the cells were held at -70 mV (the holding potential), and the potassium currents were elicited with the application of 10 mV step pulses, ranging between -70 and +80 mV. A concentration of 15.3 μM SF was used to determine the effect of SF on the activation kinetic curves, since at this concentration I_k was inhibited ~50% (close to IC₅₀). For the regression analysis of the activation kinetic curves of I_k, I_k was converted into conductance using the following equation as previously described by Wang *et al* (16): $G = I/(V - V_{rev})$, where G represents conductance, V represents the membrane potential, V_{rev} represents the reversal potential and I is the K⁺ current. The normalized conductance was shown using the following Boltzmann equation: $G/G_{max} = \{1 + \exp[(V_{1/2} - V)/k]\}^{-1}$, where G/G_{max} represents normalized conductance, V_{1/2} represents

membrane potential at half-activation, and k represents slope factor.

In order to determine the inactivation kinetic curves of I_k, cells were held at -70 mV (the holding potential) and the potassium currents were elicited with a test pulse to +40 mV, following a prepulse of between -60 and +80 mV, in increments of 10 mV. The concentration of 15.3 μM was selected to record the effect of SF on the inactivation kinetic curves of I_k, as aforementioned. For the regression analysis of inactivation kinetic curves, the steady-state inactivation curves were shown by the following Boltzmann equation: $I/I_{max} = \{1 + \exp[(V_{1/2} - V)/k]\}^{-1}$, where I/I_{max} represents the normalized currents, V_{1/2} represents the membrane potential at half-inactivation and k represents the slope factor.

Recordings were only performed from cells when the seal resistance was >1 GΩ. Five kHz was automatically obtained and set for the current recordings. When the currents reached equilibrium (≥200 sec), the extracellular solution was replaced with the extracellular solution containing drug using 40 μl solution via 4 pipette tips of the NPC-16 patch liner.

Data analysis. Whole-cell data files were imported into Igor Pro (WaveMetrics, Inc., Portland, OR, USA). Results are presented as the mean ± standard error of the mean. Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) and Sigmaplot (SPSS, Inc., Chicago, IL, USA) software were used for analysis or display. One-way analysis of variance and the Student's t-test were used to compare the difference of drug effects. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of SF on the I_k of PC12 cells. The holding potential was set to -70 mV and a 30 msec conditioning depolarization to -40 mV followed (to inactivate the Na⁺ channel) and 10-mV step pulses (200 msec duration) between -60 mV and +40 mV were applied. As shown in Fig. 1, compared with the control, the peak amplitudes of I_k were decreased following the application of SF in a concentration-dependent manner. The half inhibitory concentration (IC₅₀) and the Hill coefficient of SF were 14.9 and 0.87 μM, respectively.

Effect of SF on the activation kinetics of I_k. The effect of SF (15.3 μM) on the activation kinetics of I_k is shown in Fig. 2. The currents were induced during repeated sweeps to +80 mV from a holding potential of -70 mV, in increments of 10 mV. Following the application of 15.3 μM SF, the induced I_k was inhibited (Fig. 2A and B). The curves were fitted with the Boltzmann equation (r=0.997) and the values of V_{1/2} for activation of I_k changed from 11.2±1.8 mV (absence of SF) to 18.9±2.1 mV (n=5; P<0.01), and the slope factor k of the G/G_{max} curves showed no significant difference prior to and following the drug treatment (from 17.3±1.6 to 17.8±1.4 mV; n=5, P>0.05; Fig. 2C). Compared with the control, SF shifted the activation kinetic curve to a positive potential and had no effect on the slope factor.

Effect of SF on the inactivation kinetics of I_k. The effect of SF (15.3 μM) on the inactivation kinetic curves of I_k is shown in Fig. 3. The holding potential was set to -70 mV and the currents

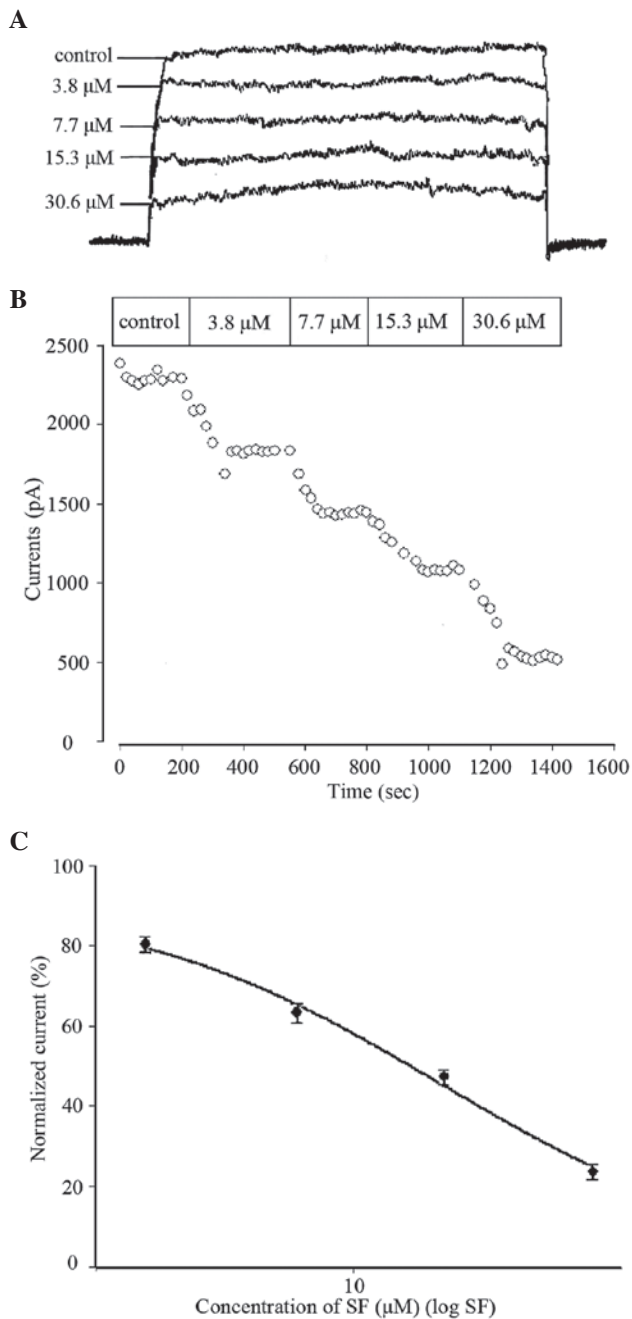


Figure 1. Effect of SF on the Ik in PC12 cells. Ik was recorded from PC12 cells using an automatic patch clamp. (A) The Ik in the absence (control) and presence of four concentrations of SF (3.8, 7.7, 15.3 and 30.6 μM). (B) The peak amplitudes of Ik were decreased with increasing SF concentrations. Four concentrations of SF were respectively applied to the patched cell in a cumulative manner in the representative time trace of Ik. (C) Concentration-normalized current following treatment with 3.8, 7.7, 15.3 and 30.6 μM SF. Each concentration of SF was applied once for ≥300s until currents reached equilibrium. SF, sodium ferulate; Ik, delayed rectifier K⁺ current.

were induced with a test pulse to +40 mV, during repeated sweeps to +80 mV from a prepulse of -60 mV, in increments of 10 mV. The curves were fitted with the Boltzmann equation ($r=0.997$). Following the application of 15.3 μM SF, the elicited Ik was inhibited (Fig. 3A) and the values of V_{1/2} for the inactivation of Ik changed from 6.7 ± 1.8 mV (absence of SF) to 1.2 ± 1.7 mV ($n=6$, $P<0.01$), and the slope factor k of the I/I_{max} curves prior to and following application of SF

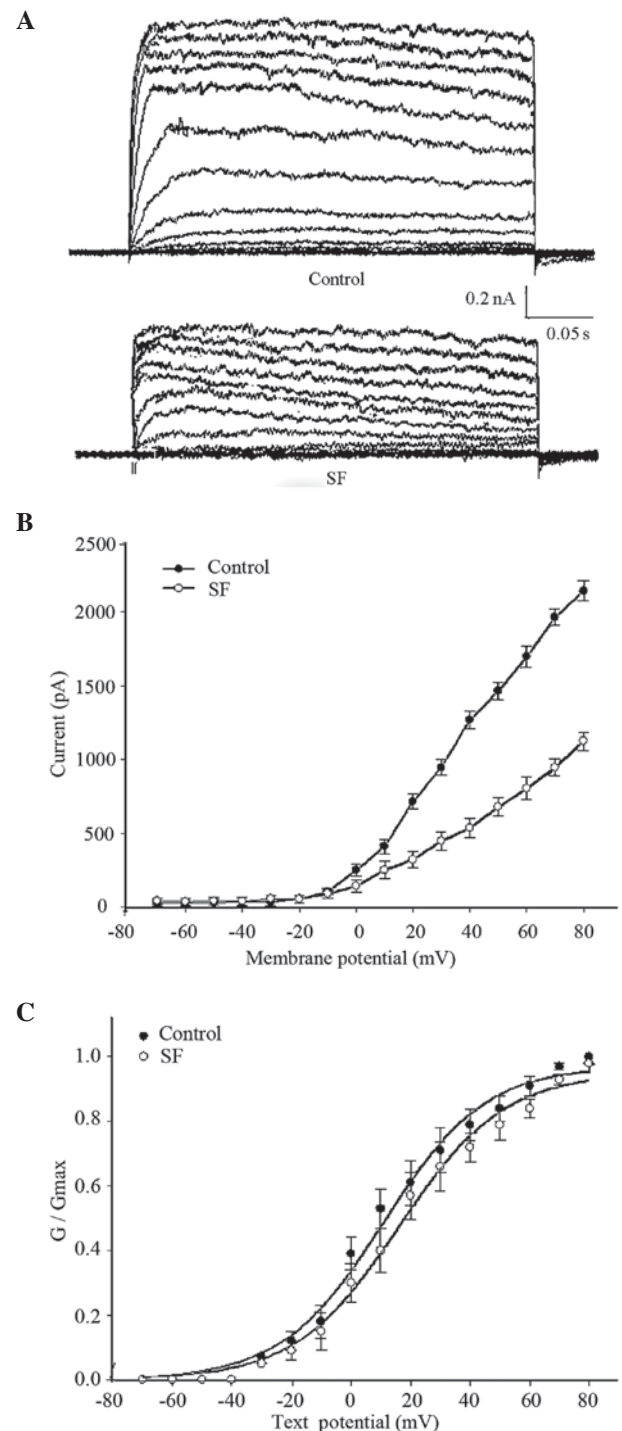


Figure 2. Effect of SF (15.3 μM) on the activation kinetics of Ik. (A) Cells were held at -70 mV, and currents were induced following step voltage pulses, ranging between -70 and +80 mV, in increments of 10 mV. Superimposed Ik responses to the application of step voltage pulses (in the absence of SF) and superimposed Ik responses to the application of step voltage pulses following the application of 15.3 μM SF. (B) The current-voltage (I-V) association of Ik in the absence (control) and following (SF) application of 15.3 μM SF. I-V curves were calculated by plotting peak current amplitude of Ik against the test potentials. (C) The steady-state activation kinetic curves for Ik in the absence (control) and following (SF) application of 15.3 μM SF. The data is presented as G/G_{max} against the test potentials and fitted with a Boltzmann equation $G/G_{max} = \{1 + \exp[(V_{1/2}-V)/k]\}^{-1}$. SF, sodium ferulate; Ik, delayed rectifier K⁺ current.

was -20.7 ± 1.4 and -21.1 ± 1.9 mV, respectively ($n=5$; $P>0.05$; Fig. 3B). Compared with the control, SF shifted the inactiva-

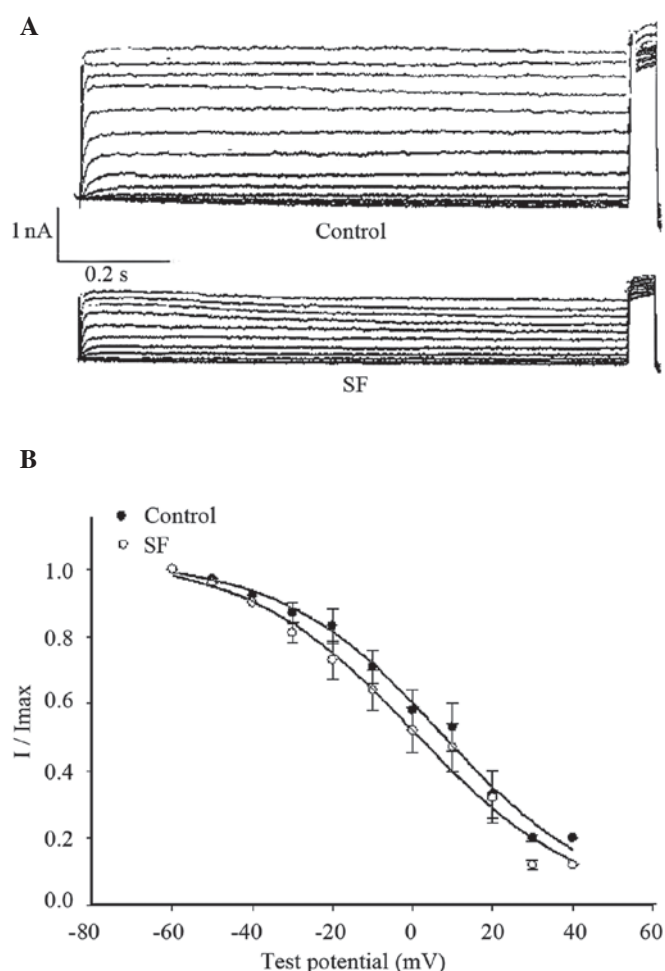


Figure 3. Effect of SF (15.3 μ M) on inactivation kinetics of I_k . (A) Cells were held at -70 mV and the currents were elicited with a test pulse to +40 mV, during repeated sweeps to +80 mV from a prepulse of -60 mV, in increments of 10 mV. Superimposed I_k responses to the application of step voltage pulses (in the absence of SF) and following application of 15.3 μ M SF. (B) I_k steady-state inactivation kinetic curves in the absence (control) and presence (SF) of 15.3 μ M SF. The data was shown by normalizing the test current amplitudes by taking the maximum value under each condition, ($I/I_{\max} = \{1 + \exp[(V_{1/2} - V)/k]\}$), as unity. SF, sodium ferulate; I_k , delayed rectifier K^+ current.

tion kinetic curve to a negative potential and had no effect on the slope factor.

Discussion

The identification of novel anti-nociceptive drugs from natural products has been the focus of numerous studies. The effect of ferulic acid-type organic acids following extraction and purification has been of particular interest. A number of papers have reported that ferulic acid acts as an antinociceptive agent (3,17,18); however, whether the K^+ channel is involved in the anti-nociceptive mechanism remains to be elucidated. As important physiological regulators, K_v channels regulate the action potential shape, membrane potentials and firing adaptation in excitable tissues, including nociceptive sensory neurons (4,5,19,20). K^+ channel alterations may reflect the clinical antinociceptive action. A number of studies have

shown that voltage-gated K^+ channels have a major role in the regulation of neuronal excitability (21,22).

In the present study, the effect of SF on voltage-gated K^+ channels in PC12 cells was analyzed using the automated patch-clamp method. The results indicate that SF treatment decreases the I_k of PC12 cells, which suggests that the voltage-activated delayed rectifier K^+ channel was inhibited following SF treatment.

The analysis of the activation and inactivation kinetic curves of I_k demonstrated that the SF affects the kinetics of activation and inactivation. The activation kinetic curve of I_k shifted to positive potentials and the value of $V_{1/2}$ increased following the application of SF, which indicates that the activation of the K^+ channels was delayed. The inactivation kinetic curve of I_k shifted to negative potentials and the value of $V_{1/2}$ decreased following application of SF. It is known that the inactivation of voltage-activated delayed rectifier K^+ channels occurs during the process of membrane repolarization in which the I_k , an outward current, decreases gradually during the membrane repolarization process (the voltage value decreases gradually from high to low). Therefore, by analyzing the inactivation kinetic curves of I_k , the reduction in the $V_{1/2}$ following the application of SF could be explained by the fact that the inactivation of the K^+ channels was delayed. The delayed activation kinetics may be the major cause for the decrease of I_k following SF treatment. The delayed activation kinetics and inactivation kinetics of I_k may be involved in the analgesic effect of SF, which suggests that the change resulted from the function of the K^+ channel or its properties.

Generally, it is thought that the inhibition of potassium currents leads to membrane excitation; however, certain studies have reported that I_k is inhibited following anesthetic and analgesic drug application (23,24). It has been hypothesized that excitation of neurons results in overall depression of potassium currents. It has also been suggested that the neuronal depression or excitation caused by inhibition of the K^+ channel is determined by the location of the K^+ channel within a neuronal network, which may also modulate the antinociceptive response (23). In the present study, it was found that SF shifted the activation kinetic curve of the delayed rectifier K^+ channel to higher membrane potentials and the inactivation kinetic curve of the delayed rectifier K^+ channel to lower membrane potential. This indicates that the delayed activation kinetics result in a delayed process of repolarization, and the delayed inactivation kinetics may result in an increase of K^+ efflux from the cell, which may be associated with the cell membrane hyperpolarization and lead to neuronal depression. This may help to explain the association between the anti-nociceptive effect of SF and the inhibition of delayed rectifier K^+ channels.

The association between the inhibition of the delayed rectifier K^+ channel and the anti-nociceptive effect of SF requires further investigation. Furthermore, as a result of the complicated association between the K^+ channel, the Ca^{2+} channel and the Na^+ channel (25,26), the effects of SF on the Ca^{2+} channel, the Na^+ channel and their associations also require further investigation, which is likely to explain the mechanism of the anti-nociceptive effect of SF. In conclusion, SF treatment significantly inhibits the delayed rectifier K^+

currents of PC12 cells in a concentration-dependent manner. The mechanism may be associated with the delayed activation and inactivation of the I_K channel.

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