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Inhibitory action of L-type Ca^{2+} current by paeoniflorin, a major constituent of peony root, in NG108-15 neuronal cells

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Abstract

The effects of paeoniflorin, a glycoside isolated from the root of *Paeonia lactiflora*, on ion currents in a mouse neuroblastoma and rat glioma hybrid cell line, NG108-15 were investigated. Paeoniflorin (1–300 μM) reversibly produced an inhibition of L-type voltage-dependent Ca^{2+} current ($I_{Ca,L}$) in a concentration-dependent manner. Paeoniflorin caused no change in the overall shape of the current–voltage relationship of $I_{Ca,L}$. The IC_{50} value of paeoniflorin-induced inhibition of $I_{Ca,L}$ was 14 μM. However, neither adenosine deaminase (1 U/ml) nor 8-cyclopentyl-1, 3-dipropylxanthine (10 μM) could reverse the inhibition by paeoniflorin of $I_{Ca,L}$. Paeoniflorin (30 μM) shifted the steady-state inactivation curve of $I_{Ca,L}$ to more negative membrane potentials by approximately −10 mV. It also prolonged the recovery of $I_{Ca,L}$. The inhibitory effect of paeoniflorin on $I_{Ca,L}$ exhibited tonic and use-dependent characteristics. Paeoniflorin could effectively suppress $I_{Ca,L}$ evoked by action potential waveforms. Paeoniflorin at a concentration of 30 μM produce a slight inhibition of voltage-dependent Na^+ current and delayed rectifier K^+ current. Under current-clamp configuration, unlike adenosine, this compound decreased the firing of action potentials. Taken together, this study indicates that paeoniflorin can block L-type Ca^{2+} channels in NG108-15 cells in a mechanism unlinked to the binding to adenosine receptors. The effects of paeoniflorin on ion currents may partly, if not entirely, contribute to the underlying mechanisms through which it affects neuronal or neuroendocrine function.

Keywords: Paeoniflorin; L-type Ca^{2+} current; NG108-15 cells

1. Introduction

Paeoniflorin is a bioactive monoterpene glucoside in *Paeoniae radix*, the roots of *Paeonia lactiflora* (Ranunculaceae) (Shibata et al., 1963). *P. radix* has been used to regulate menstrual flow for the treatment of menstrual disorders and to relieve spasmodic abdominal pain and muscle stiffness. A previous study showed the inhibitory effect of *P. radix* extract on pentylenetetrazol-induced EEG power spectrum changes and suggested that *P. radix* had anticonvulsant activity (Sugaya et al., 1991). Its inhibition of the seizure-related decrease of extracellular Ca^{2+} and consequent intracellular Ca^{2+} increase might contribute to its anticonvulsant action. In addition, paeoniflorin has been reported to be a cognitive enhancer and to attenuate learning impairment in aged rats (Ohta et al., 1994; Watanabe, 1997; Tabata et al., 2001). The effect of paeoniflorin to ameliorate memory disruption and to reverse guanethidine-induced hypotension might be associated with its binding to adenosine A_1 receptor (Tabata et al., 2001; Cheng et al., 1999). It has also been shown to bind to adenosine A_1 receptors to induce the translocation of protein kinase C and glucose transporter in isolated rat adipocytes (Lai et al., 1998). This compound might induce the release of adenosine from isolated rat white adipocytes (Tang et al., 2003).

Paeoniflorin has been shown to attenuate the contraction induced by veratrine in isolated atria and aorta of rats (Tsai et al., 1997, 1999). This compound could also inhibit the acetylcholine-induced phasic contraction in isolated rat uterus (Tsai et al., 2001). It has been shown to attenuate high K^+-induced tonic contraction in isolated mouse vas deferens (Chen et al., 2002). Taken together, these findings suggested that paeoniflorin might be able to suppress voltage-dependent Ca^{2+} channels. Furthermore, it has been recently demonstrated that age-related memory impairment could coincide with increases in the expression of L-type Ca^{2+} channel protein α-subunit (Cav1.3) (Veng et al.,

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2003). However, to our knowledge, the effects of paeoniflorin on ion currents have not been extensively studied, although a recent report showed that paeoniflorin could block Na^+ current in mouse hippocampal CA_1 neurons (Zhang et al., 2003).

Previous reports have revealed that the major component of voltage-dependent Ca^{2+} currents in undifferentiated NG108-15 cells is the dihydropyridine-sensitive L-type Ca^{2+} channel (Connor and Henderson, 1997; Wu et al., 2002). In addition to exhibiting the activity of delayed rectifier K^+ channels (Wu et al., 2001), these cells have been shown to express the Cav1.3 (α_{1D}) Ca^{2+} channel-forming subunit (Kim et al., 1998). Therefore, in this study, we attempted to determine the mechanism by which paeoniflorin interacts with ion currents (i.e., L-type Ca^{2+} current [$I_{Ca,L}$], Na^+ current [I_{Na}], and delayed rectifier K^+ current [$I_{K(DR)}$]) to cause any effects on NG108-15 neuronal cells.

2. Materials and methods

2.1. Cell preparation

The clonal strain NG108-15 cell line, originally formed by Sendai virus-induced fusion of the mouse neuroblastoma clone N18TG-2 and the rat glioma clone C6 BV-1, was obtained from the European Collection of Cell Cultures (ECACC-88112302; Wiltshire, UK). Cells were grown in monolayer culture in 50-ml plastic culture flasks in a humidified environment of 5% CO_2/95% air at 37 °C. Cells were maintained at a density of 10^6/ml in 5 ml of Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (v/v), and 2 mM L-glutamine. Experiments were performed 5 or 6 days after cells were subcultured (60–80% confluence).

2.2. Electrophysiological measurements

Before each experiment, cells were dissociated, and an aliquot of cell suspension was transferred to a recording chamber positioned on the stage of an inverted microscope (DM IL; Leica Microsystems, Wetzlar, Germany). Cells were bathed at room temperature (20–25 °C) in normal Tyrode's solution containing 1.8 mM $CaCl_2$. The recording pipettes were pulled from thin-walled borosilicate glass capillaries (Kimax-51; Kimble Glass, Vineland, NJ, USA) using a two-stage microelectrode puller (PP-830; Narishige, Tokyo, Japan) and the tips were fire-polished with a microforge (MF-83; Narishige). When filled with pipette solution, their resistance ranged between 3 and 5 MΩ. Ion currents were recorded in tight-seal whole cell recordings by means of an RK-400 patch-clamp amplifier (Bio-Logic, Claix, France) (Wu et al., 2001).

2.3. Data recording and analysis

The signals were displayed on an analog/digital oscilloscope (HM 507; Hameg Inc., East Meadow, NY, USA) and on a liquid crystal projector (PJ550-2; ViewSonic Corporation, Walnut, CA, USA). The data were on-line stored in a Pentium III-grade computer (Slimnote VX_3; Lemel, Taipei, Taiwan) via a universal serial bus port at 10 kHz through a high-speed/low-noise analog/digital interface (Digidata 1322A; Axon Instruments, Union City, CA, USA). This device was controlled by commercially available software (pCLAMP 9.0; Axon Instruments). Currents were low-pass filtered at 1 or 3 kHz. Ion currents recorded during whole cell experiments were stored without leakage correction and analyzed subsequently using the pCLAMP 9.0 software (Axon Instruments), the Origin 6.0

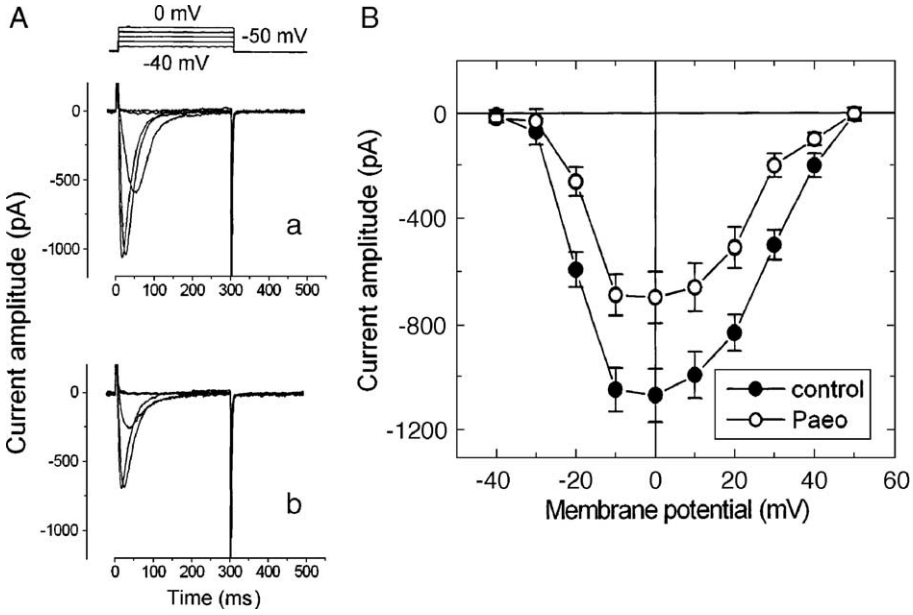

Fig. 1. Inhibitory effect of paeoniflorin on I–V relations of $I_{Ca,L}$ in NG108-15 cells. Cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$, 10 mM tetraethylammonium chloride, and 1 μM tetrodotoxin. (A) Superimposed current traces in the absence (a) and presence (b) of 10 μM paeoniflorin. The cell was depolarized from −50 to various potentials ranging from −40 to 0 mV in 10 mV increments. The uppermost part indicates the voltage protocol used. (B) Averaged I–V relations of $I_{Ca,L}$ obtained in the absence (●) and presence (○) of 10 μM paeoniflorin (Paeo). Each point represents the mean±S.E.M. (n=7–11).

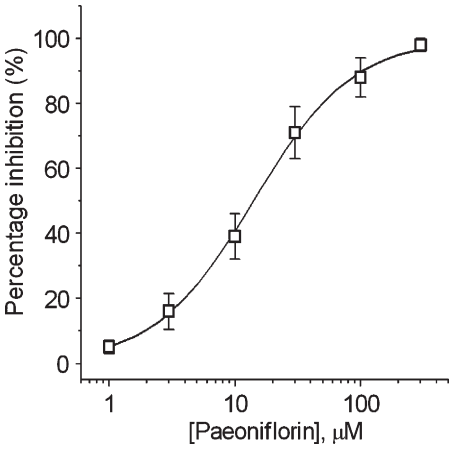

Fig. 2. Concentration–response curve for paeoniflorin-induced inhibition of $I_{Ca,L}$ in NG108-15 cells. Each cell was depolarized from −50 to 0 mV with a duration of 300 ms. The amplitude of $I_{Ca,L}$ obtained after addition of paeoniflorin was compared with the control value, i.e., in the absence of paeoniflorin (mean±S.E.M., n=5–9 for each point). The smooth line represents the best fit to a Hill function. The vales for IC_{50}, maximally inhibited percentage of $I_{Ca,L}$ and the Hill coefficient were 14 μM, 99% and 1.1, respectively.

software (Microcal Software, Inc., Northampton, MA, USA), SigmaPlot 7.0 software (SPSS, Inc., Apex, NC, USA), or custom-made macros in Excel (Microsoft, Redmont, WA, USA). The pCLAMP-generated voltage-step protocols were used to examine the current–voltage (I–V) relations for ion currents. In some experiments, action potentials recorded from the cells that were bathed in normal Tyrode's solution were replayed to elicit $I_{Ca,L}$. Action potential waveform-evoked capacitative and leakage currents were determined by applying a negative going action potential waveform and subtracted from total membrane current to yield the voltage-dependent Ca^{2+} current (Lo et al., 2001).

To calculate the percentage inhibition of paeoniflorin, each cell was depolarized to 0 mV from a holding potential of −50 mV, and the amplitude of $I_{Ca,L}$ after application of paeoniflorin (1–300 μM) was compared with the control value. The concentration-dependent effect of paeoniflorin on the inhibition of $I_{Ca,L}$ was fitted to a Hill function with a non-linear least-squares fitting algorithm. That is, percentage inhibition = $(E_{max}\times[C]^{n}_{H})/(IC_{50}{}^{n}_{H}+[C]^{n}_{H})$, where $[C]$ represents the concentration of paeoniflorin; IC_{50} and n_H are the paeoniflorin concentration required for a 50% inhibition and the Hill coefficient, respectively; E_{max} is the paeoniflorin-induced maximal inhibition of $I_{Ca,L}$. The time constants for activation (τ_{act}) and inactivation (τ_{inact}) of $I_{Ca,L}$ obtained in the absence and presence of paeoniflorin were calculated by fitting currents to a single- or two-exponential function.

All values are reported as means±S.E.M. and error bars are plotted as S.E.M. The paired or unpaired Student's t test and one-way analysis of variance with a least-significance difference method for multiple comparisons was used for the statistical evaluation of differences among means. A value of $P<0.05$ was considered to be statistically significant.

2.4. Drugs and solutions

Paeoniflorin was obtained from the Pharmaceutical Industry Technology and Development Center (Taipei, Taiwan), tetrodotoxin was from Alomone Labs (Jerusalem, Israel). 18α-

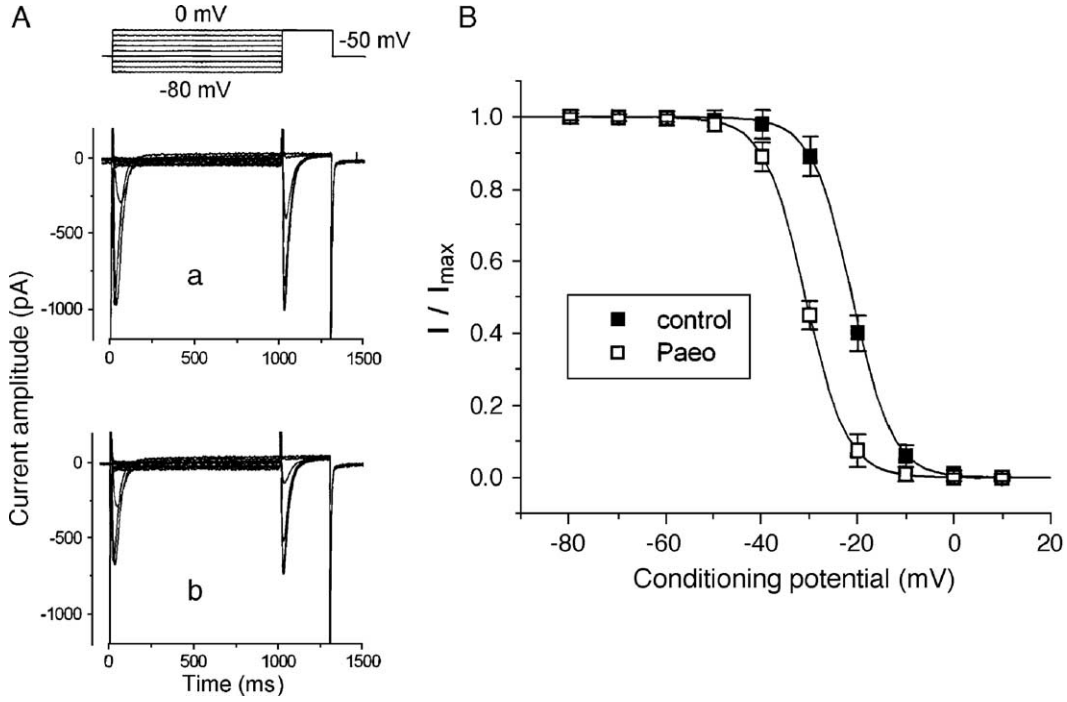

Fig. 3. Steady-state inactivation of $I_{Ca,L}$ in the absence and presence of paeoniflorin. By the use of a double-pulse protocol, the steady-state inactivation parameters of $I_{Ca,L}$ were determined. (A) Superimposed current traces obtained in the absence (a) and presence (b) of 10 μM paeoniflorin. The uppermost part indicates the voltage protocol used. (B) Normalized amplitude of $I_{Ca,L}$ (I/I_{max}) constructed against the conditioning potentials. The curves were well fit by the Boltzmann equation (see text in details). Mean±S.E.M. (n=4–5). ▒: control; □: 10 μM paeoniflorin (Paeo).

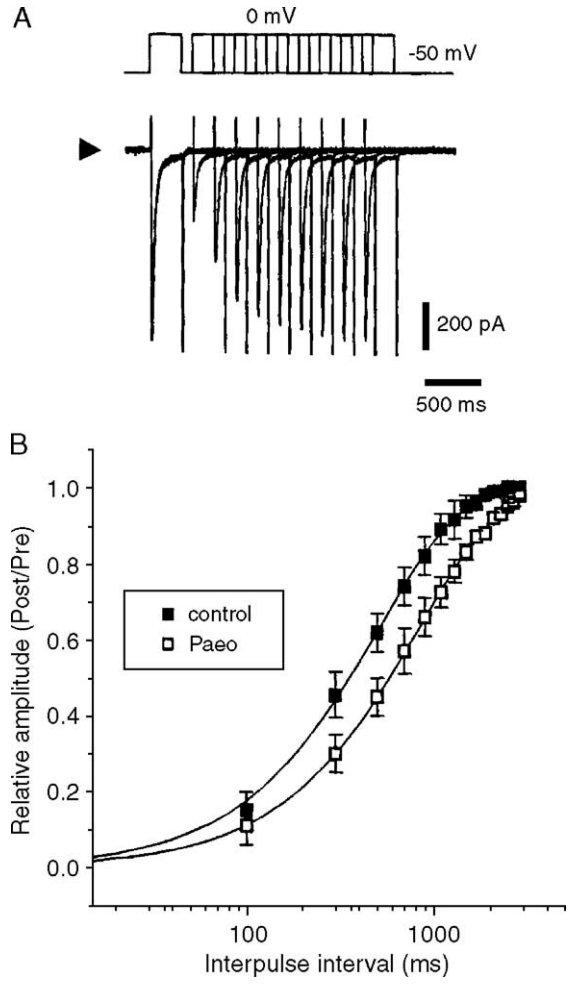

Fig. 4. Effect of paeoniflorin on the time course of recovery from the inactivation of $I_{Ca,L}$ in NG108-15 cells. Cells, bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$, were depolarized from −50 to 0 mV with a duration of 300 ms, and various interpulse durations were applied. Normalized currents taken before (pre) and after (post) the interpulse interval are plotted versus different interpulse intervals. An example of current traces obtained by a double-pulse protocol in the control is illustrated in (A). Arrowhead indicates the zero current level. The upper part denotes the voltage protocol used. (B) Time course of recovery from inactivation of $I_{Ca,L}$ with or without application of 10 μM paeoniflorin (Paeo). Each point represents the mean±S.E.M. (n=4–6).

Glycyrrhetinic acid was obtained from Aldrich Chemical (Milwaukee, WI, USA), and S (+) SDZ-202 791 was from Biomol (Plymouth Meeting, PA, USA). Tissue culture media, L-glutamine, penicillin–streptomycin, fungizone, and trypsin were obtained from Life Technologies (Grand Island, NY, USA). Paeoniflorin was dissolved in dimethyul sulfoxide (less than 0.01%) and made immediately prior to experiments. Drugs were applied through perfusion or added to the bath to obtain the final concentration indicated.

The composition of normal Tyrode's solution was as follows (in mM): NaCl 136.5, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5 and Hepes–NaOH buffer 5.5 (pH 7.4). To record membrane potential or K^+ current, the patch pipettes were filled with a solution (in mM): K-aspartate 130, KCl 20, KH_2PO_4 1, $MgCl_2$ 1, EGTA 0.1, Na_2ATP 3, Na_2GTP 0.1 and Hepes–KOH buffer 5 (pH 7.2). To measure Ca^{2+} current, K^+ ions inside the pipette solution were replaced with equimolar Cs^+ ions, and the pH was adjusted to 7.2 with CsOH.

3. Results

3.1. Effect of paeoniflorin on voltage-dependent L-type Ca^{2+} current ($I_{Ca,L}$) in NG108-15 cells

The whole-cell configuration of the patch-clamp technique was used to investigate the effect of paeoniflorin on ion currents in NG108-15 cells. In these experiments, cells were bathed in normal Tyrode's solution that contained 1.8 mM $CaCl_2$. As shown in Fig. 1, the effect of paeoniflorin on $I_{Ca,L}$ was examined at different membrane potentials and an I–V relationship of $I_{Ca,L}$ was constructed. By comparing these two I–V curves, the exposure to paeoniflorin (10 μM) was found to suppress $I_{Ca,L}$ significantly without a change in the overall shape of the I–V curves for $I_{Ca,L}$. The inhibitory effect of paeoniflorin on $I_{Ca,L}$ was completely reversible after the washout of paeoniflorin. A further application of S (+) SDZ-202 791 (3 μM) could reverse the inhibition of $I_{Ca,L}$ caused by paeoniflorin at various membrane potentials examined (data not shown). S (+) SDZ-202 791 is an opener of L-type Ca^{2+} channels (Triggle and Rampe, 1989). When cells were depolarized from −50 to 0 mV, paeoniflorin (10 μM) significantly decreased the amplitude of $I_{Ca,L}$ from 1066±102

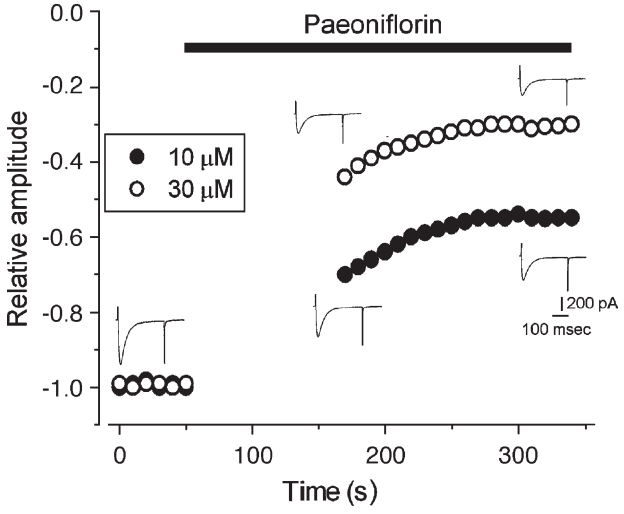

Fig. 5. Tonic and use-dependent block of $I_{Ca,L}$ by paeoniflorin in NG108-15 cells. In these experiments, the cell was held at −50 mV, and the depolarizing pulse from −50 to 0 mV (300 ms in duration) was applied at 0.1 Hz. The application of paeoniflorin is denoted by a horizontal bar. The alteration of the relative amplitude of $I_{Ca,L}$ during cell exposure to various concentrations of paeoniflorin (10 and 30 μM) is illustrated. The amplitude of $I_{Ca,L}$ in the control was taken as −1.0. Immediately after the depolarizing pulses were stopped, various concentrations of paeoniflorin were added to the bath. The repetitive depolarizing pulses to 0 mV at 0.1 Hz were applied again 2 min after the cessation of voltage pulses, but in continued presence of paeoniflorin (10 and 30 μM). ●: paeoniflorin (10 μM); ○: paeoniflorin (30 μM). Note that in the presence of paeoniflorin, the amplitude of $I_{Ca,L}$ evoked by the first depolarizing step following a long pause had already been suppressed (i.e., tonic block), and during the repetitive stimuli, the amplitude of $I_{Ca,L}$ decreased in an exponential manner (i.e., use-dependent block).

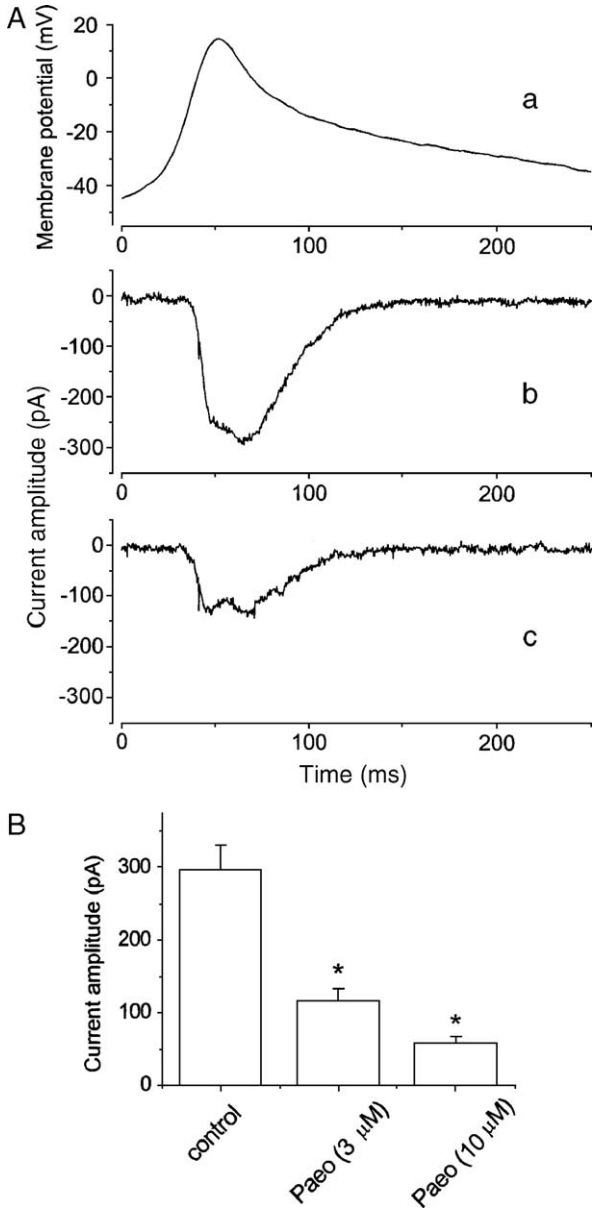

Fig. 6. Inhibitory effect of paeoniflorin on $I_{Ca,L}$ in response to action potential waveforms in NG108-15 cells. Trace a shown in (A) is the original waveform of action potentials in an NG108-15 cell obtained under current-clamp condition. Current traces shown in (b) and (c) are action potential waveform-evoked $I_{Ca,L}$ in the control, and $I_{Ca,L}$ in response to an action potential waveform obtained 1 min after the application of 3 μM paeoniflorin, respectively. (B) Bar graph showing the effect of paeoniflorin (3 and 10 μM) on the amplitude of $I_{Ca,L}$ evoked by action potential waveforms. Mean±S.E.M. (n=5–6). *Significantly different from control.

to 697±97 pA (n=6). At the same depolarizing protocol, paeoniflorin (10 μM) caused no significant change in the kinetics of either activation or inactivation of $I_{Ca,L}$ [control: τ_{act}=4±1 ms, $\tau_{inact(f)}$=13±4 ms, $\tau_{inact(s)}$=62±7 ms; paeoniflorin: τ_{act}=4±1 ms, $\tau_{inact(f)}$=14±4 ms, $\tau_{inact(s)}$=64±8 (n=6)].

The relationship between the concentration of paeoniflorin and the percentage inhibition of $I_{Ca,L}$ is illustrated in Fig. 2. paeoniflorin (1–300 μM) inhibited the amplitude of $I_{Ca,L}$ in a concentration-dependent manner. The half-maximal concentration required for the inhibitory effect of paeoniflorin was 14 and 300 μM almost fully suppressed the amplitude of $I_{Ca,L}$. These results demonstrate that paeoniflorin produces a concentration-dependent inhibition of $I_{Ca,L}$ in NG108-15 cells.

3.2. Voltage-dependence of paeoniflorin-induced inhibition of $I_{Ca,L}$

To characterize the inhibitory effects of paeoniflorin on $I_{Ca,L}$, we also examined the voltage-dependence of the effect of paeoniflorin on $I_{Ca,L}$ in NG108-15 cells. Fig. 3 shows the steady-state inactivation curve of $I_{Ca,L}$ obtained in the absence and presence of paeoniflorin (10 μM). In these experiments, the interval between two sets of voltage pulses was 60 s to avoid incomplete recovery of $I_{Ca,L}$. As shown in Fig. 3, the relationships between conditioning potentials and the normalized amplitudes of $I_{Ca,L}$ in the absence and presence of

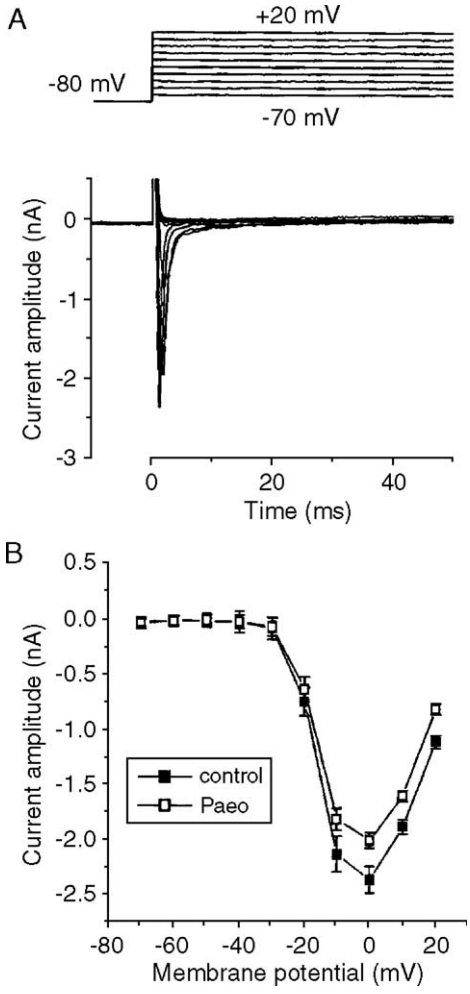

Fig. 7. Effect of paeoniflorin on I_{Na} in NG108-15 cells. In these experiments, cells were bathed in Ca^{2+}-free Tyrode's solution containing $CdCl_2$ (0.5 mM) and tetraethylammonium chloride (10 mM). Cells were depolarized from a holding potential of −80 mV to various potentials ranging from −70 to +20 mV in 10 mV increments. Superimposed current traces obtained in the control are illustrated in (A). The upper part indicates the voltage protocol used. (B) Averaged I–V relations of I_{Na} in the absence (▒) and presence (□) of 30 μM paeoniflorin (Paeo). Mean±S.E.M. (n=4–7).

paeoniflorin were fitted to a Boltzmann function using the least-squares method: $I=I_{max}/\{1+\exp[(V-a)/b]\}$, where I_{max} is the maximal activated $I_{Ca,L}$, V is the membrane potential in mV, a is the membrane potential for half-maximal inactivation, and b is the slope factor of the inactivation curve. In the absence of paeoniflorin, $a=-21.2\pm1.6$ mV and $b=4.2\pm0.4$ mV ($n=5$), whereas in the presence of paeoniflorin (10 μM), $a=-30.9\pm1.4$ mV and $b=4.3\pm0.4$ mV (n =5). Thus, paeoniflorin not simply reduced the maximal availability of L-type Ca^{2+} channel, but also shifted the inactivation curve to a hyperpolarized potential (9.7±0.5 mV, $n=6$). However, we found no significant change in the slope of the curve during the exposure to paeoniflorin. The results indicate that paeoniflorin can suppress the amplitude of $I_{Ca,L}$ in a voltage-dependent manner in NG108-15 cells.

3.3. Effect of paeoniflorin on the recovery of $I_{Ca,L}$ from inactivation

The effect of paeoniflorin on the recovery of $I_{Ca,L}$ from inactivation was also studied using a double-pulse protocol. The recovery of $I_{Ca,L}$ from inactivation at a holding potential of −50 mV was examined at different times with a test step (0 mV for 300 ms). As shown in Fig. 4, in the control, the amplitude of $I_{Ca,L}$ almost completely recovered from inactivation when the recovery time was 2 s. The time course of recovery from inactivation in the control could be fitted to a single-exponential function with a time constant of 0.51±0.04 s ($n=5$). However, during cell exposure to 10 μM paeoniflorin, the recovery from inactivation was significantly prolonged with a time constant of 0.83±0.05 s ($n=5$) (Fig. 4). After a 2-s interval, the amplitude of $I_{Ca,L}$ was found to recover completely from inactivation in the control; however, in the presence of paeoniflorin, a substantial block of $I_{Ca,L}$ was still observed. Therefore, paeoniflorin could produce a prolongation of the recovery from inactivation of $I_{Ca,L}$ in NG108-15 cells.

3.4. Use-dependence of paeoniflorin-induced inhibition of $I_{Ca,L}$

Another series of experiments was performed to determine the use-dependent characteristics of paeoniflorin-induced block of $I_{Ca,L}$. As illustrated in Fig. 5, when the depolarizing pulses from −50 to 0 mV (300 ms in duration) were applied at 0.1 Hz, no decline in the amplitude of $I_{Ca,L}$ at this stimulation protocol could be observed for more than 5 min. When the amplitude of $I_{Ca,L}$ remained constant, the depolarizing pulses were stopped, and paeoniflorin at a concentration of 10 or 30 μM was thereafter applied to the bath. After 2 min of cessation, the depolarizing pulses from −50 to 0 mV at 0.1 Hz were given to the cell again. The relative amplitude of $I_{Ca,L}$ with respect to that before the addition of paeoniflorin was plotted in Fig. 5. Interestingly, the inhibition by paeoniflorin of $I_{Ca,L}$ in NG108-15 cells was found to consist of both tonic and use-dependent components. For example, when cells were exposed to 10 μM paeoniflorin, after a 2-min pause, the amplitude of $I_{Ca,L}$ elicited by the first voltage step was suppressed by 69±6% ($n=5$) (i.e., tonic inhibition). Moreover, following the repetitive stimuli, the amplitude of $I_{Ca,L}$ could further be reduced to a constant level in an exponential fashion (i.e., use-dependent inhibition). The percentage inhibition of $I_{Ca,L}$ following the repetitive stimuli was increased to 41±5% ($n=5$). When the paeoniflorin concentration was increased to 30 μM, the amplitude of $I_{Ca,L}$ at

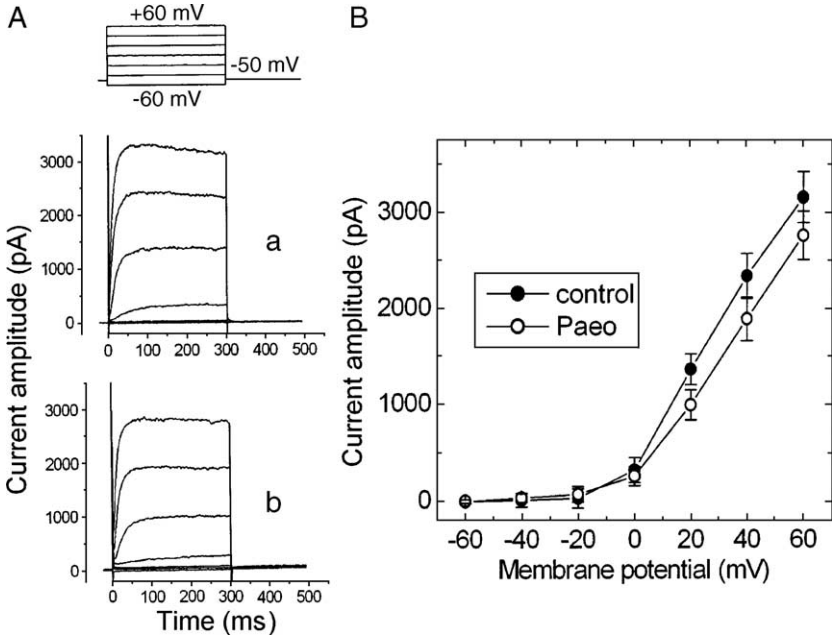

Fig. 8. Effect of paeoniflorin on delayed rectifier K^+ current ($I_{K(DR)}$) in NG108-15 cells. Cells were bathed in Ca^{2+}-free Tyrode's solution containing tetrodotoxin (1 μM) and $CdCl_2$ (0.5 mM). (A) Superimposed current traces for $I_{K(DR)}$ obtained in the absence (a) and presence (b) of 30 μM paeoniflorin. The cell was depolarized from −50 mV to various potentials ranging from −60 to +60 mV in 20 mV increments. The uppermost part indicates the voltage protocol used. (B) Averaged I–V relations of $I_{K(DR)}$ obtained with or without application of 30 μM paeoniflorin (Paeo). ●: control; ○: paeoniflorin (30 μM). Mean±S.E.M. ($n=4$–8).

the first depolarizing pulse and during subsequent repetitive stimuli were significantly decreased by 41±5 and 28±5% (n=5), respectively. These results can be interpreted to indicate that the inhibition of $I_{Ca,L}$ caused by paeoniflorin observed in NG108-15 cells is composed of two components (i.e., tonic and use-dependent components).

3.5. Effect of paeoniflorin on $I_{Ca,L}$ in response to action potential waveforms in NG108-15 cells

The size and time course of $I_{Ca,L}$ in response to change in an action potential waveform has been previously described to be distinguishable from that during a rectangular voltage-clamp pulse (Lo et al., 2001). Therefore, the effect of paeoniflorin on action potential waveform-evoked $I_{Ca,L}$ was investigated. In this series of experiments, the original waveforms of action potentials that were obtained from other cells were digitally used to generate the templates. These voltage-clamp templates were then replayed to evoke $I_{Ca,L}$. Consistent with previous observations in pituitary GH_3 cells (Lo et al., 2001), $I_{Ca,L}$ elicited by an action potential waveform had a characteristic biphasic form in NG108-15 cells (Fig. 6). The early component of $I_{Ca,L}$ was coincident with the rising phase of the action potential, whereas a second, late component of $I_{Ca,L}$ appeared during the falling phase of the action potential waveform. As illustrated in Fig. 6, the amplitude of $I_{Ca,L}$ in response to action potential waveforms was reduced in the presence of paeoniflorin. After application of paeoniflorin (10 μM), $I_{Ca,L}$ evoked by action potential waveforms was significantly suppressed by 19±5% [from 297±34 to 58±9 pA (n=6)]. However, paeoniflorin (10 μM) inhibited $I_{Ca,L}$ in response to rectangular depolarizing pulses only by 40%. Therefore, by comparison, $I_{Ca,L}$ evoked by action potential waveforms appears to be more sensitive to inhibition by paeoniflorin than that during rectangular steps.

3.6. Effect of paeoniflorin on voltage-dependent Na^+ current (I_{Na}) and delayed rectifier K^+ current ($I_{K(DR)}$) in NG108-15 cells

A previous study has shown the ability of paeoniflorin to suppress I_{Na} in hippocampal neurons (Zhang et al., 2003). We also examined whether paeoniflorin could have any effect on I_{Na} in these cells. The experiments were performed when cells were bathed in Ca^{2+}-free Tyrode's solution containing $CdCl_2$ (0.5 mM) and tetraethylammonium chloride (10 mM). The cells were held at −80 mV, and depolarizing pulses (50 ms in duration) to different potentials ranging from −70 to +20 mV in 10-mV increment) were applied. Paeoniflorin (30 μM) caused a slight inhibition of I_{Na} in NG108-15 cells (Fig. 7). When cells were exposed to paeoniflorin (30 μM), current amplitude, determined at the level of 0 mV, was significantly decreased to 2.01±0.07 nA from a control value of 2.35±0.12 nA (n=5). However, no change in I–V relations of I_{Na} present in these cells can be demonstrated in the presence of paeoniflorin.

We also examined the effect of paeoniflorin on $I_{K(DR)}$ that was previously described in these cells (Wu et al., 2001). In these experiments, cells were bathed in Ca^{2+}-free Tyrode's solution containing 1 μM tetrodotoxin and 0.5 mM $CdCl_2$. When the cell was held at −50 mV and various potentials ranging from −40 to +60 mV at 0.1 Hz were applied, paeoniflorin (30 μM) slightly suppressed the amplitude of $I_{K(DR)}$ (Fig. 8). For example, when the depolarizing pulses from −50 to +60 mV, paeoniflorin (30 μM) decreased current amplitude at the end of the voltage pulses from 3152±265 to 2757±251 pA (n=6).

3.7. Effect of paeoniflorin on spontaneous action potentials in NG108-15 cells

In a final series of experiments, the effect of paeoniflorin on repetitive firing of action potentials was investigated. Cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Current-clamp configuration was performed with a K^+-containing pipette solution. The typical effect of paeoniflorin on spontaneous action potentials in these cells is illustrated in Fig. 9A. When cells were exposed to paeoniflorin, the repetitive firing of action potentials was readily reduced (Fig. 9). For example, paeoniflorin at a concentration of 10 μM significantly

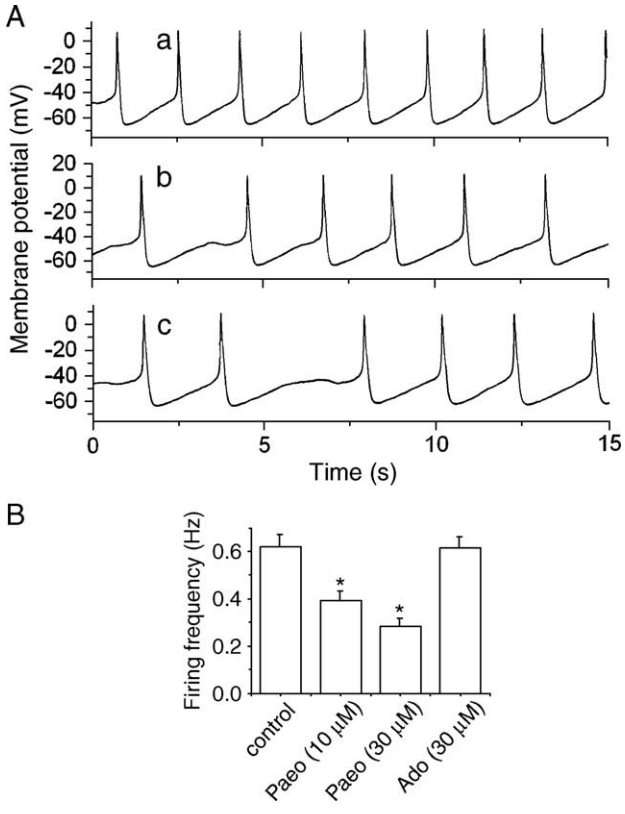

Fig. 9. Effect of paeoniflorin on the firing of action potentials in NG108-15 cells. Cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Patch pipettes were filled with a K^+-containing solution. Membrane potential was measured under current-clamp condition. (A) Original potential traces showing the effect of paeoniflorin on spontaneous action potentials. Trace (a) is the control, and traces (b) and (c) were obtained 2 min after application of 10 and 30 μM paeoniflorin, respectively. (B) Bar graph showing the summary of the effect of paeoniflorin (Paeo) and adenosine (Ado) on spontaneous action potentials. Each point represents the mean±SEM (n=4–6). *Significantly different from control.

decreased the frequency from 0.62±0.05 to 0.39±0.04 Hz (n=5). However, adenosine (30 μM) had little or no effect on spontaneous action potentials. Therefore, the paeoniflorin-induced reduction of firing frequency could be primarily explained by its blockade of $I_{Ca,L}$.

4. Discussion

The major findings of this study are as follows. First, in NG108-15 neuronal cells, paeoniflorin inhibits the amplitude of $I_{Ca,L}$ in a concentration-dependent manner. Second, paeoniflorin can cause a negative shift in the steady-state inactivation curve of $I_{Ca,L}$. Third, paeoniflorin prolongs the recovery of $I_{Ca,L}$ inactivation. Fourth, the inhibitory effect of paeoniflorin on $I_{Ca,L}$ appears to have both tonic and use-dependent components. Fifth, paeoniflorin at a concentration of 30 μM has a slight inhibition of I_{Na} and $I_{K(DR)}$. These results suggest that the inhibition by paeoniflorin of these channels can be one of ionic mechanisms underlying the paeoniflorin-induced change in functional activity of neurons or neuroendocrine cells.

In this study, we found that when NG108-15 cells were exposed to paeoniflorin, there was a significant prolongation of the recovery from $I_{Ca,L}$ inactivation. The recovery from inactivation appeared to be slower in the presence of paeoniflorin. Furthermore, paeoniflorin could produce a distinct shift in the steady state inactivation curve of $I_{Ca,L}$. It is thus likely that paeoniflorin binds to the inactivated state of L-type Ca^{2+} channels. Importantly, the ability of paeoniflorin to produce a negative shift in the inactivation curve suggest that paeoniflorin-induced block of $I_{Ca,L}$ is voltage-dependent. These observations are consistent with previous reports showing inability of paeoniflorin to cause any effects on the basal tone of isolated aortic rings and vas deferens (Tsai et al., 1999; Chen et al., 2002). It is apparent that the sensitivity of $I_{Ca,L}$ to paeoniflorin in neurons depends not only on the concentration of paeoniflorin, but also on the preexisting level of resting potential, if similar results are found in intact brain neurons.

Paeoniflorin could attenuate high K^+-induced tonic contraction in isolated vas deferens (Chen et al., 2002). In this study, we provide the direct evidence that paeoniflorin has a depressant action on $I_{Ca,L}$ in NG108-15 cells. The IC_{50} value of paeoniflorin required for the inhibition of $I_{Ca,L}$ was 14 μM. $I_{Ca,L}$ in response to action potential waveforms was more sensitive to block by paeoniflorin than that evoked by rectangular steps. In mouse hippocampal CA_1 neurons, paeoniflorin inhibited I_{Na} with an IC_{50} value of 271 μM (Zhang et al., 2003). In our study, we also found that paeoniflorin at a concentration of 30 μM produced a slight inhibition of I_{Na} and $I_{K(DR)}$. Therefore, L-type Ca^{2+} channels functionally expressed in neurons or neuroendocrine cells are likely to be the targets of this compound. In addition, paeoniflorin, similar to phenylalkylamines, was found to exhibit use-dependent block of $I_{Ca,L}$. The inhibition of $I_{Ca,L}$ caused by paeoniflorin consisted of tonic and use-dependent components. Therefore, it is possible that when action potentials in neurons in vivo fired more frequently, the inhibitory action of paeoniflorin on $I_{Ca,L}$ would be enhanced.

It is noteworthy that paeoniflorin produces varying effects on contractile force in different portions of isolated vas deferens (Chen et al., 2002). This difference could be due to the possibility that there is the different affinity for paeoniflorin to the binding site in different preparations. This effect could be possibly associated with block of $I_{K(DR)}$ in the presence of paeoniflorin at a concentration greater than 30 μM. However, the ability of paeoniflorin to antagonize veratrine-induced increase in contractile force of vas deferens and isolated aortic rings (Tsai et al., 1999; Chen et al., 2002) can be primarily explained by its block of $I_{Ca,L}$. A recent report showed that Cav1.3 channel trafficking could be involved in glucose desensitization of pancreatic β-cells. It will be of interest to determine whether paeoniflorin induced decrease in blood sugar as described previously (Hsu et al., 1997) is associated with its block of $I_{Ca,L}$ in pancreatic β-cells.

In our study, glycyrrhetinic acid, a main component of *Glycyrrhizae radix*, had little or no effect on $I_{Ca,L}$ (data not shown). Glycyrrhetinic acid and paeoniflorin were reported to be main constituents in Huangqin-Tang (Zuo et al., 2003). It remains to be clarified whether paeoflorin contained in Huangqin-Tang interacts with the Ca^{2+} channel to affect biological function and to exert neuroprotective effects (Sugaya et al., 1991; Tsuda et al., 1997).

In summary, results from the present study indicate that the major part of paeoniflorin-mediated inhibition of Ca^{2+} current in NG108-15 cells was primarily due to the blockade of L-type Ca^{2+} channels, although paeoniflorin is structurally unique and distinct from prototypical blockers of $I_{Ca,L}$. The inhibitory effect of paeoniflorin is not associated with its binding to adenosine receptors. Paeoniflorin appears to be an interesting tool used to isolated L-type components of I_{Ca} in excitable cells.

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