Blockade of paeoniflorin on sodium current in mouse hippocampal CA$_1$ neurons

ZHANG Guang-Qin, HAO Xue-Mei, CHEN Shi-Zhong[1], ZHOU Pei-Ai, CHENG He-Ping, WU Cai-Hong[2]

State Key Laboratory of Biomembrane and Membrane Biotechnology, College of Life Sciences, Peking University, Beijing 100871;
[1]Department of Traditional Chinese Medicine, School of Pharmaceutical Science, Peking University, Beijing 100083, China

ABSTRACT

AIM: To study the blockade of paeoniflorin (Pae) on I_{Na} in the acutely isolated hippocampus neurons of mice. **METHODS:** The whole-cell patch clamp technique was used. **RESULTS:** Pae inhibited I_{Na} in frequency-dependent and concentration-dependent manners, with an IC$_{50}$ of 271 µmol/L. Pae 0.3 mmol/L shifted the activation potential of the maximal I_{Na} from -40 mV to -30 mV, shifted the steady-state activation and inactivation curves toward more positive and negative potentials by10.8 mV, and 18.2 mV, respectively, and postponed the recovery of I_{Na} inactivation state from (4.2±0.7) ms to (9.8±1.2) ms. **CONCLUSION:** Pae inhibited I_{Na} in mouse hippocampus neurons.

INTRODUCTION

Paeoniflorin (Pae) is a active compound extracted from *Paeonia lactiflora Pall* stems. It can ameliorate learning and memory impairment in rodents and spatial cognitive impairment caused by cholinergic dysfunction in rat. Moreover, Pae can also attenuate learning impairment of aged rats, inhibit calcium overloading injury in cultured primary cortex neurons, and protect neuron damage in the hippocampus induced by the cobalt focus epilepsy model[1-5]. It was reported that total paeony glycoside had protective effects on local cerebral ischemia and acute complete cerebral ischemia induced by clipping three arteries in rat[6, 7]. But its mechanism is still unclear.

Brain damage caused by cerebral ischemia or hypoxia induces the accumulation of cytosol Na$^+$ which leads to Ca^{2+} overload by activation of Na$^+$-Ca^{2+} exchange. Therefore, Na$^+$ channel plays an important role in regulating the sustained Na$^+$ influx during ischemia/hypoxia. Blocking of Na$^+$ channel is expected to exert neuroprotection. Many studies have demonstrated that sodium channel blockers have protective effect against cerebral ischemia[8-10]. So, we investigated the effect of Pae on Na$^+$ channels to probe into its possible mechanisms of neuroprotection.

MATERIALS AND METHODS

Cell preparation and solutions All experiments were performed on hippocampal pyramidal cells which were acutely isolated from 10-16 d old Kunming mice according to the method described previously[11]. Briefly, hippocampal CA$_1$ region was cut into pieces of about 0.4 mm×0.4 mm and incubated at 32 ºC for 2 h in artificial cerebrospinal solution (ACS) containing: NaCl 124, KCl 5, K$_2$HPO$_4$ 1.2, MgSO$_4$ 1.3, CaCl$_2$ 2.4, NaHCO$_3$ 26, glucose 10 mmol/L, pH 7.4. Then they were transferred into ACS containing protease 1.5 g/L at 32 ºC for 40 min, bubbled with 95 % O$_2$-5% CO$_2$.

The digested issue were washed three times with ACS and dispersed by pipettes. The cells were transferred into a 35-mm culture dish filled with 2 mL normal extracelluar solution containing: NaCl 150, KCl 5, CaCl$_2$ 2.6, MgCl$_2$ 1.1, Hepes 10 mmol /L, pH 7.4 .

Sodium current recording method Whole-cell currents were recorded with a patch-clamp amplifier (Axopatch 200B, Axon Instruments, USA). The data was digitized at 5 kHz and filtered at 3 kHz, then stored in a PC 486 computer using pCLAMP software (Ver 6.02, Axon Instrument, USA). The pipettes were pulled in two steps from borosilicate glass capillaries with 1.5 mm outer diameter. The pipette had tip resistance of 2-4 MΩ when filled with intracellular solution: CsCl 75, CsF 75, Creatine Phosphate-Tris 14, TEA-Cl 2, MgCl$_2$ 2, Hepes (sodium salt) 10, egtazic acid 2.5, Na$_2$ATP 3 mmol/L, adjusted to pH 7.4 with CsOH. All experiments were performed at room temperature (20 °C-25 °C).

Experimental protocols To eliminate Ca^{2+} currents, 0.2 mmol/L CdCl$_2$ was added into the external solution. Currents were elicited by 20-ms depolarizing pulses to potentials ranging from -90 mV to +70 mV in 10 mV increment at a rate of 0.25 Hz. The holding potential (HP) was -100 mV. The peak current at each potential was normalized to the membrane conductance. The conductance was calculated from the peak value of I_{Na} at each depolarizing potential using the equation: $G=I/(V-V_{rev})$, where G is the membrane conductance at the potential V, I is the peak current, and V_{rev} is the reversal potential.

The steady-state activation curve of I_{Na} was well fitted by the Boltzmann equation: $G/G_{max}=1/\{1+\exp[(V_h-V)/k]\}$, where G_{max} is the maximal conductance, V_h is the half activation voltage, and k is the slope factor.

The steady-state inactivation of I_{Na} was investigated with a double pulse protocol. Superimposed Na$^+$ current traces were evoked with a 20-ms test pulse depolarized to -40 mV from the holding potential of -100 mV, preceding with 200-ms conditional pulses depolarized to various potential levels from -120 mV to 20 mV in 10 mV steps. The steady-state inactivation curve was obtained by normalizing the current values using the peak I_{Na} at -120 mV as unity. The inactivation curve was fitted by the Boltzmann equation: $I/I_{max}=1/\{1+\exp[(V-V_h)/k]\}$, where I is the peak current evoked from the conditional potential V, I_{max} is the maximal peak current, V_h is the half-inactivation voltage and k is a slope factor.

The time course of I_{Na} recovery from the steady state inactivation was studied using a standard two-pulse protocol. A condition pulse depolarized to -40 mV from a holding potential of -100 mV for 50-ms was followed by various recovery periods (0 to 60 ms) and then by a test pulse steped to -40 mV for 50 ms. The recovery process of I_{Na} could be fitted by a single exponential function.

Date analysis and drug applications All the data were analyzed by the pCLAMP 6.02 (Axon Instrument, USA) and Sigmaplot (Jandel Scientific, USA) software. Statistical analysis was made by Student's paired *t*-test and the data were expressed as mean±SD. Pae was a gift from School of Pharmaceutical Science, Peking University. A 50 mmol/L stock solution of Pae was made and was added to the extracellular solution.

RESULTS

Blockade of Pae on I_{Na} An inward currents was activated by 20-ms depolarizing pulses from -90 to +70 mV in 10 mV step at 0.25 Hz, with a holding potential of -100 mV. It could be blocked completely and reversibly by bath application of TTX 1 µmol/L. Therefore, this inward current was the TTX-sensitive sodium current (I_{Na}).

The concentration-response curve of Pae on I_{Na} was examined by a 20-ms pulse depolarized to -40 mV after the application of Pae. I_{Na} values relative to the control was plotted against the drug concentrations. The data were fitted well with logistic equation $Y=1/[1+(IC_{50}/C)^n]$, where C is the concentration of the drug, Y and n are the fraction of the maximum inhibition percentage and the Hill coefficient. The suppressive effect of Pae on I_{Na} was concentration-dependent in the range of 10-1000 µmol/L with IC$_{50}$ of 271 µmol/L (95 % confidence limits: 206-342). The Hill constant was 0.995. The maximal suppression of Pae 10, 30, 100, 300, and 1000 µmol/L on I_{Na} were 3.4 %±1.2 %, 8 %±4 %, 28 %±8 %, 54 %±9 %, and 78 %±9 %, respectively (Fig 1A).

In the control experiments, the amplitude of I_{Na} had no change over 40 min. After the addition of Pae 0.3 mmol/L, I_{Na} showed a substantial decrease in time-dependent manner. The blockade occurred in 1-2 min after the addition of Pae, and reached to a steady state in about 10 min (*n*=8). The maximal suppression of Pae on I_{Na} was 52 %±8 % at -40 mV. After washout of Pae with extracellular solution for 10 min, I_{Na} could par-

A

B

C
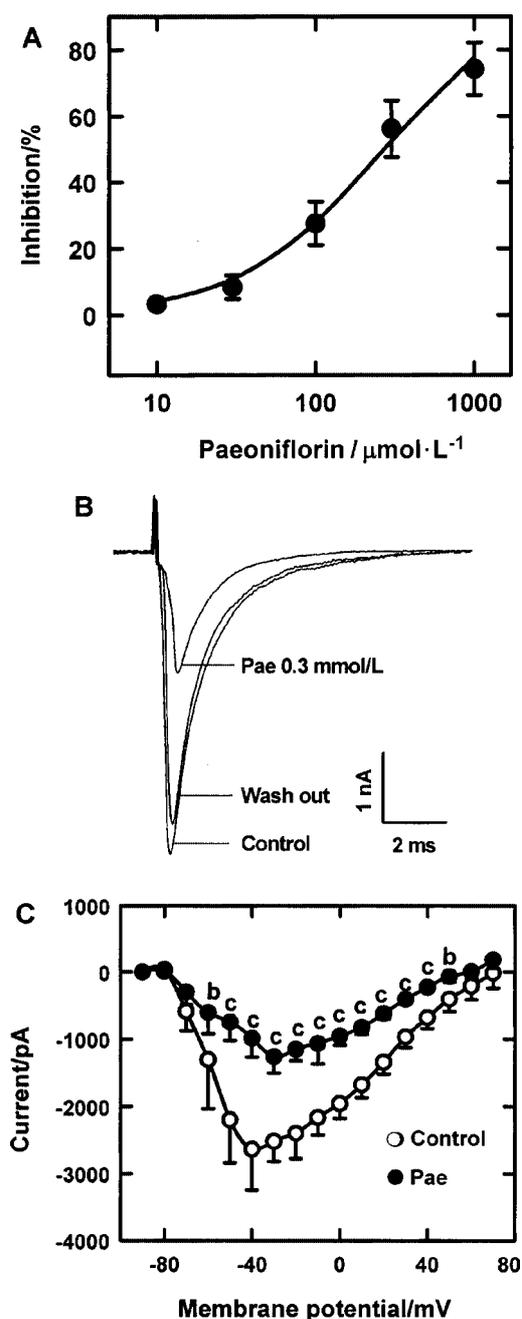
Fig 1. Effect of Pae on I_{Na}. A) Cencentration-response curve of Pae on I_{Na}. B) Current traces of I_{Na} obtained in the absence and presence of Pae 0.3 mmol/L. C) Current-voltage (*I-V*) relationship of I_{Na}. n =8. Mean±SD. [b]$P<0.05$, [c]$P<0.01$ vs control.

tially recovered (Fig 1B).

Effect of Pae on the current-voltage relationship (*I-V*) of I_{Na} Current traces were elicited by 20-ms pulses depolarized to +70 mV from -90 mV with a 10 mV step. Under the control condition, I_{Na} was activated at -70 mV and reached maximum amplitude near -40 mV. After the application of Pae 0.3 mmol/L, the activation potential of the maximal I_{Na} was shifted from -40 mV to -30 mV (Fig 1C).

Effect of Pae on the activation and inactivation kinetics of I_{Na} The activation curves of I_{Na} before and after the application of Pae were obtained from the data of *I-V* curves. The V_h values were (-54.9±2.7) mV and (-44.1±2.5) mV ($P<0.01$, n=8), respectively, with k value of (-7.0±0.9) mV and (-6.7±1.3) mV ($P>0.05$, n=8). Pae shifted the steady-state activation curves of I_{Na} towards more positive potential without changing the slope factor.

After exposure to Pae 0.3 mmol/L, the steady-state inactivation curve was shifted toward more negative potentials. The V_h and k of I_{Na} were (-63±3) mV and (7.9±1.8) mV in control; while (-81±4) mV ($P<0.01$) and (10.8±1.3) mV ($P<0.05$) in the presence of Pae (n=8), respectively (Fig 2A).

Effect of Pae on the recovery kinetics of I_{Na} In the presence of Pae 0.3 mmol/L, the time course of recovery from inactivation was obviously increased, the time constants of recovery curves were (4.2±0.7) ms in control and (9.8±1.2) ms in the presence of Pae ($P<0.01$, n=8), respectively (Fig 2B).

Frequency-dependent effect of Pae Frequency-dependent effect of Pae 0.3 mmol/L was studied by 30 pulses depolarized to -40 mV from a holding potential of -100 mV at different frequencies (1, 2, 4 Hz). Under the control condition, the responses of I_{Na} to depolarizing pulses remained unchanged. After the application of Pae, the suppression of Pae on I_{Na} was enhanced along with the increase of the stimuli frequency. Thus, Pae showed a frequency-dependent effect on I_{Na}. When stimulated at the rate of 4 Hz, the inhibition reached to the maximum value. The curve of normalized current amplitudes could be well fitted with a single-exponential equation (Fig 2C).

DISCUSSION

The present results indicated that Pae inhibited I_{Na} in concentration-dependent and frequency-dependent manners. The activation potential of the maximal I_{Na} shifted toward more positive potential by 10 mV. The activation and inactivation curves of I_{Na} shifted toward more positive and negative potential respectively, which resulted in reduction of the steady state window current. The time constant of recovery from the inactivation state were markedly increased. These data suggest that
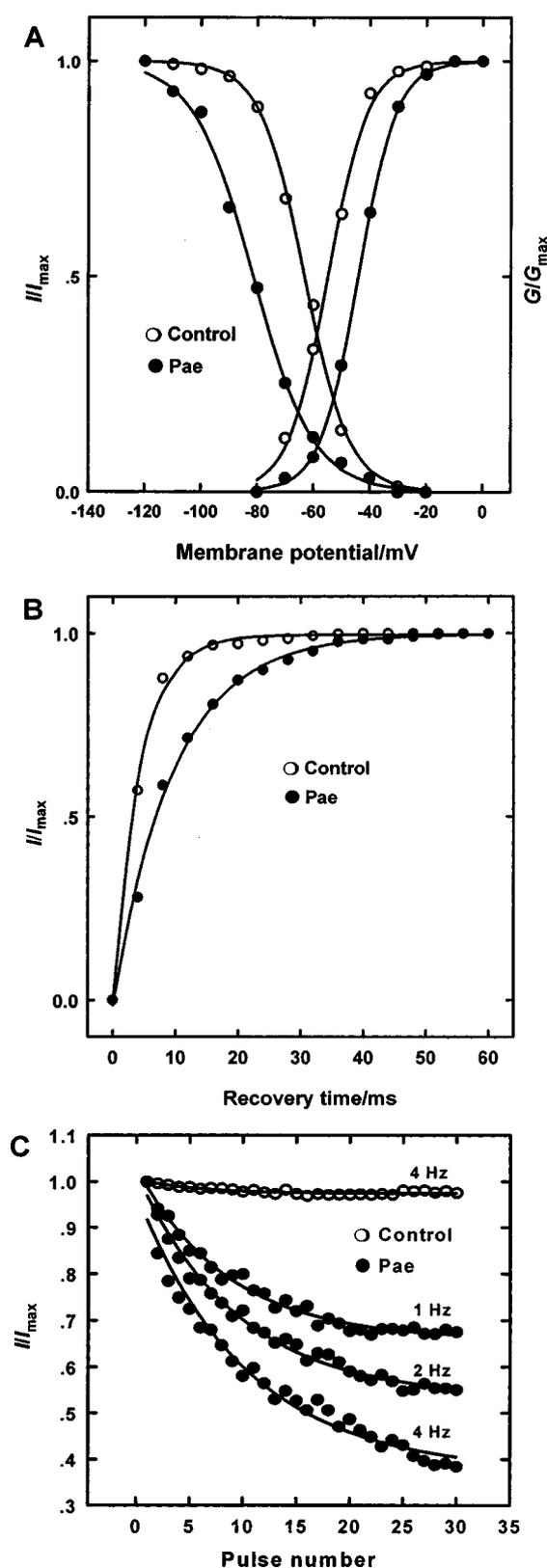
Fig 2. Effect of Pae 0.3 mmol/L on kinetics and frequency-dependence of I_{Na}. A) The steady state activation and inactivation curves of I_{Na}. n=8 cells. B) The recovery curve of I_{Na} from steady state inactivation. n=8 cells. C) Frequency-dependent suppression of Pae on I_{Na}. n=6 cells.

Pae not only inhibits I_{Na}, but also alters the gating properties of the sodium channels.

In our experiment, the inhibitory effect of Pae occurred in 1-2 min and reached to the maximal steady value in about 10 min. These results showed that the time course of Pae to suppress I_{Na} maximally was relatively slower than that of some sodium channel blockers such as TTX. We speculated that Pae might indirectly interact with the Na^+ channel via regulating phosphorylation of the channel proteins. The voltage-gated Na^+ channels can be phosphorylated by cAMP-dependent protein kinase (PKA) and PKC. The activation of PKA and PKC result in the decrease of I_{Na} and the change of channel kinetics[12]. Therefore, the inhibitory effect of Pae on I_{Na} may depend on channels phosphorylation by activating PKA and PKC.

Many findings indicated the Pae could prevent brain damage[4-7]. But the mechanism of its protective effects on ischemia, especially on the ion channels, is still unclear. Previous studies have demonstrated that the influx of Na^+ and overload of Ca^{2+} induced by Na^+-Ca^{2+} exchange contributed to brain injury during ischemia/hypoxia. Blocking these channels can improve post-ischemia functional and mechanical recovery[13]. Recently, there is more evidence showing that Na^+ channels blockers may exert neuroprotective effect in models of brain ischemia. So, blocking Na^+ channels is considered as a target for protecting brain damage. Compared with common Na^+ channel blockers such as lidocaine, mexiletine and phenytoin, Pae displays similar potency in various experimental models of cerebral ischemia[5-7]. Hence, the neuroprotective effect of Pae maybe attribute to the inhibition of sodium channels. These findings also offer evidence for potential use of Pae to treat brain ischemia in clinical practice.

In summary, the blocking effect of Pae on I_{Na} in hippocampal CA_1 neurons may be one of the mechanisms of the protection from brain hypoxia or ischemia.

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