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Puerarin inhibits tetrodotoxin-resistant sodium current in rat dorsal root ganglion neurons

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KEY WORDS puerarin; sodium channels; tetrodotoxin; spinal ganglia; patch-clamp techniques

AIM: To test if puerarin (Pue) affects slow sodium current in dorsal root ganglion (DRG) neurons. **METHODS**: Tetrodotoxin resistant (TTXr) sodium current was recorded with whole cell patch clamp technique on DRG neurons of young adult rats. **RESULTS**: Pue 0.01 - 2 mmol · L^{-1} inhibited TTXr sodium current by 9.5 % - 83.2 %. The inhibition was concentration-dependent and partially reversible, but was not use-dependent nor voltage-dependent. Pue did not affect the inactivation but changed the potential for half maximal conductance from -26 mV to -16 mV, suggesting the activation process was inhibited. **CONCLUSION**: Pue moderately inhibits TTXr sodium current of rat DRG neurons.

Sodium ion influx via slow sodium channels may be related to hypoxic and ischemic damages in heart and brain, while sodium channel blockers may have protective actions[1-4]. Puerarin (Pue), an isoflavone compound isolated from *Pueraria lobata* (Willd), has been used for the treatment of cardiovascular diseases due to its hypotensive action, anti-myocardial ischemia and anti-arrhythmia effects by lowering myocardial excitability[5-7]. Therefore, the protective effects of Pue may be a result of Na^+ channel blocking and, if so, may also be used in the protection of brain. To test this possibility, we have chosen dorsal root ganglion (DRG) cells as a neuronal model of slow sodium current because DRG neurons are endowed with tetrodotoxin-resistant (TTXr) sodium channels with a characteristic kinetics of slow inactivation[8-9].

MATERIALS AND METHODS

Isolation of DRG neurons Young adult ♀ Wistar rats (weight 83 ± 21 g) were killed by stunning and cervical dislocation. Dissected ganglia were minced and incubated at 37 ℃ in a solution of collagenase (Sigma) 1.2 g · L^{-1} and trypsin (type III, Sigma) 1 g · L^{-1} in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline for 50 - 60 min. Newborn calf serum (10 %) was added as a trypsin inhibitor and incubated for 15 min. Cells were harvested with trituration and plated on glass coverslips coated with poly-*L*-lysine (Sigma), cultured in Dulbecco's modified Eagle's medium supplemented with newborn calf serum (10 %) at 37 ℃, and were used for patch clamping after 9 - 36 h of incubation.

Electrophysiological recording Sodium currents were recorded with whole-cell patch clamp technique[10]. The pipette solution contained: CsF 110, $MgCl_2$ 5, egtazic acid (EGTA) 11, NaCl 10, *N*-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid) (HEPES) 10 mmol · L^{-1}. The pH was adjusted to 7.2 by tris (hydroxymethyl) aminomethane (Tris). The external solution contained: NaCl 60, CsCl 5, choline chloride 50, tetraethylammonium (TEA) chloride 20, KCl 5, $CaCl_2$ 0.01, $MgCl_2$ 5, glucose 5, HEPES 5, Na-HEPES 5 mmol · L^{-1}. The pH was 7.4[8,9]. The electrode resistances were 1 MΩ - 3 MΩ. An Ag-AgCl pellet-internal solution-agar bridge was used as the reference electrode. The membrane currents were recorded using an Axopatch 200A amplifier (Axon Instruments, USA). pCLAMP 5.51 program was used to produce signals, collect and process data. The junction potential was corrected and series resistance was compensated by more than 70 %. The leak currents were subtracted digitally unless otherwise stated. All experiments were performed at 21 ℃ - 24 ℃. The bathing chamber, containing 0.7 mL solution, was perfused with the complete change of solution within 5 min.

Drugs Pue was supplied by the Institute of Materia Medica, Chinese Academy of Medical Sciences (purity >95 %). TTX was purchased from Sigma Co.

Analysis of data Results are expressed as $\bar{x} \pm s$. Statistical significances were analyzed by t test.

RESULTS

Isolation of TTXr sodium current Among 132 DRG neurons tested in the presence of TTX 2 μmol · L^{-1} in the bath solution, 102 cells with the diameter of 20.5 ± 2.7 μm were found to have inward current with the holding potential of -50 mV and a step depolarization to 0 mV. This current

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disappeared, or even the direction of current reversed when extracellular Na^+ was completely replaced by choline (Fig 1A). These features are in accordance with the TTXr sodium current described in the literature[8,9].

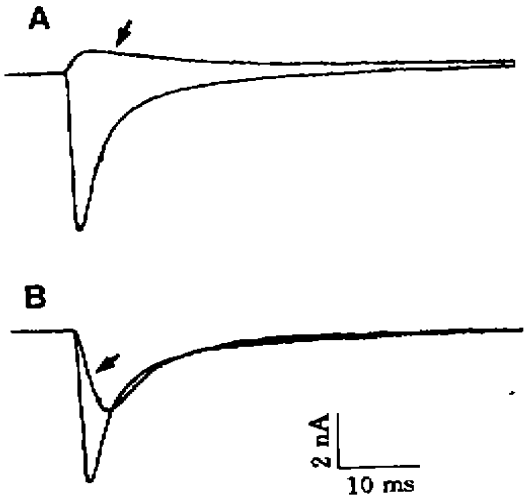

Fig 1. TTXr sodium current. A) Identified by the comparison of inward current when perfused with the standard external solution and the Na^+-free external solution (arrow). The currents were recorded in the presence of TTX 2 μmol·L^{-1}, and the potential was stepped from −50 mV to 0 mV for 50 ms. B) Before and after (arrow) addition of Pue 1 mmol·L^{-1}. The inhibition of Pue on this cell was 49 %.

Effect of Pue on sodium current Upon the addition of Pue (1 mmol·L^{-1}) to the bath, the inward current declined to 60 ± 23 % (n = 7, P < 0.05) with a delay of 1 − 2 min, and reached steady state within 5 − 8 min (Fig 1B). The time to peak of TTXr sodium current was 12.3 ± 0.8 and 13.5 ± 0.8 ms (n = 5) before and after the addition of Pue, respectively. In 7 out of 12 cells, the peak of TTXr sodium current retrieved from 38.9 % to 71.3 % (P < 0.01) of their original amplitude when Pue was washed out.

Current-voltage relationship The current-voltage relationship of TTXr sodium current in 5 cells before and after the addition of Pue 1 mmol·L^{-1} was shown in Fig 2. The membrane was held at −50 mV and inward current was evoked by step depolarization to various levels once every 60 s. The maximal activation of TTXr sodium current

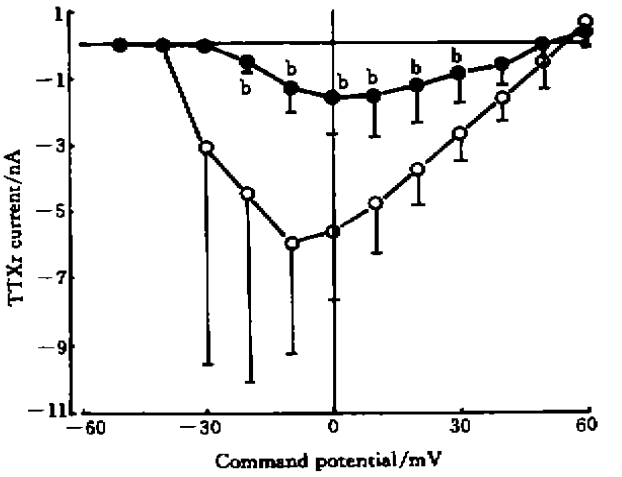

Fig 2. Relationship between peak amplitude of TTXr Na^+ current (evoked by 50 ms depolarization to various levels from a holding potential of −50 mV) and command potential. n = 5, $\bar{x} \pm s$. ^bP<0.05 *vs* control. ○ Control, ● Pue 1 mmol·L^{-1}.

appeared at −10 mV for control and 0 mV for Pue applicated. TTXr sodium current was decreased by Pue at the membrane potentials from −20 mV to 30 mV. The activation threshold potentials were −40 mV for control, and −30 mV for Pue. The mean reversal potentials were about 54 mV in both conditions.

The shift of the current-voltage relationship was seen more clearly when the sodium conductance (g) was plotted as a function of membrane potential (Fig 3) according to: $g = I/(V - V_r)$, where V_r is the reversal potential. The maximal sodium conductance was not significantly affected by Pue, but the potential which elicited half maximal conductance was estimated to be −26 mV before and −16 mV after the addition of Pue 1 mmol·L^{-1}.

Steady-state sodium current inactivation The steady-state inactivation (h_∞) curves of TTXr sodium current were measured with a 1000 ms pre-pulse in the range of −50 to 40 mV (conditioning potential), followed by a 50 ms test pulse to 0 mV (Fig 4). These currents were not leak-subtracted. The half-inactivation potential (V_h), and the slope factor of the h_∞ curve (K_h), were determined with inactivation equation:

$$I_{test}/I_{max} = 1/\{1 + \exp[V - V_h)/K_h]\}$$

In control condition (n = 3), V_h and K_h were −36.8 ± 1.8 mV and 4.0 ± 0.4, respectively. In

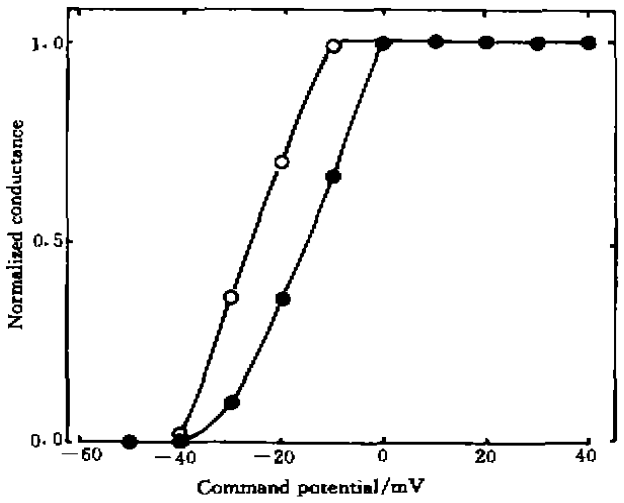

Fig 3. Conductance-voltage relationship for TTXr sodium current before and after the application of Pue 1 mmol·L^{-1}. The conductance were calculated from the data in Fig 2. $n=5$. ○ Control, ● Pue.

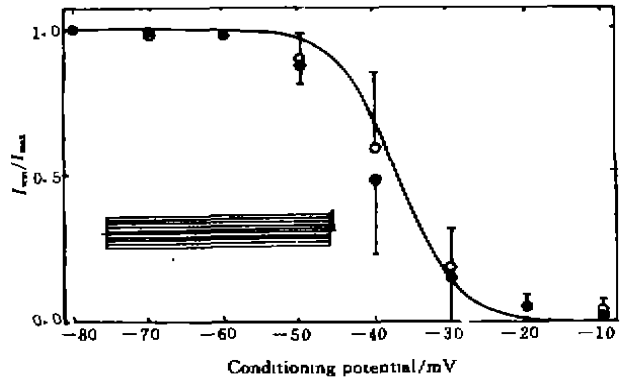

Fig 4. Effect of Pue 1 mmol·L^{-1} on steady-state inactivation for TTXr sodium current. The relative peak amplitude of TTXr sodium current during a step depolarization was plotted as a function of the conditioning potential. Two curves, fitted with the inactivation equation (see text), overlapped almost completely. $n=3$, $\bar{x}\pm s$. ○ Control, ● Pue.

the presence of Pue 1 mmol·L^{-1} ($n=3$), these parameters did not change significantly, being -37.0 ± 1.8 mV for V_h and 4.0 ± 0.4 for K_h.

Concentration-response relationships TTXr sodium current was evoked by a 50-ms step depolarization to 0 mV from a holding potential of −50 mV. The % of modified sodium currents by Pue at 0.01, 0.1, 0.2, 1, and 2 mmol·L^{-1} were 9 ± 7, 15 ± 6, 35 ± 21, 60 ± 23, and 83 ± 30, respectively (Fig 5).

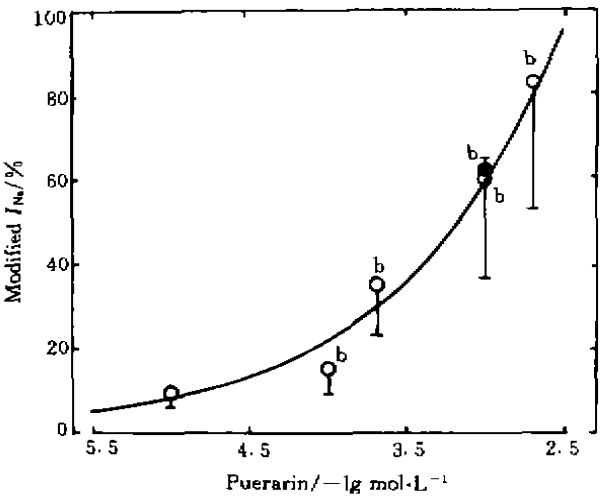

Fig 5. Concentration-dependent effect of Pue on TTXr Na$^+$ currents evoked by a 50-ms depolarization to 0 mV from a holding potential of −50 mV (○) or −80 mV (●). $n=4-7$, $\bar{x}\pm s$. $^bP<0.05$.

Use-dependent and voltage-dependent relationship TTXr sodium currents were measured at the stimulating frequencies of 1, 5, and 10 Hz before and after the addition of Pue[3]. The relative currents (I_{Na} at each stimulation compared with that of the initial one) were not significantly decreased with the application of Pue, suggesting that the effect of Pue was not use- or frequency-dependent. With Pue 1 mmol·L^{-1}, the modified sodium current percentages were 62.4 ± 2.7 % ($n=4$) at the holding potential of −80 mV (Fig 5) *vs* 60 ± 23 % at −50 mV ($P>0.05$), indicating that the inhibition of Pue on TTXr sodium current was not voltage-dependent.

DISCUSSION

Our results have shown that Pue inhibits TTXr sodium current in rat DRG neurons. Since 1) TTX sodium channels were blocked completely with 2 μmol·L^{-1} TTX, 2) the TTXr current was abolished in the Na$^+$-free external solution, and 3) F$^-$, Mg^{2+}, and egtazic acid were used in the internal and/or external solution which were known to abolish Ca^{2+} currents, the possibility that the inward current in this study might be the components of TTX-sensitive sodium current or Ca^{2+} current can be excluded[8,9].

In DRG neurons, Pue attenuated the peak

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amplitude of TTXr sodium current dose-dependently. It did not affect the kinetics of inactivation but changed the potential for half maximal conductance and the activation curve toward the depolarization direction, suggesting that the activation process was partially inhibited.

TTXr Na^+ current, with similar characteristics in electrophysiology and pharmacology, has been found to be presented in cardiac cells, sensory ganglion neurons, hippocampal and striatal neurons[1,9,11]. Several cardiovascular drugs known to modulate Na^+ channels, such as lidocaine, phenytoin and mexiletine, prevent neuronal damage in models of brain ischemia[2-4,12,13]. Evidently, increased Na^+ influx contributes to ischemic damage via a large entry of Ca^{2+} resulting from Na^+-Ca^{2+} exchange[1]. Based on the findings of this study, it appears that the effect of Pue on myocardial ischemia may be due to inhibition of TTXr sodium current. Since Pue has been used in the treatment of retinal ischemia[14], the possibility that Pue may have neuroprotective action against brain hypoxia and ischemia merits further investigation.

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葛根素抑制大鼠背根神经节细胞河豚毒素不敏感性钠电流

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关键词 葛根素；钠通道；河豚毒素；脊神经节；膜片箝技术

脑保护作用

目的：测定葛根素(Pue)对大鼠背根神经节(DRG)细胞河豚毒素不敏感性(TTXr)钠电流的作用。**方法**：采用全细胞钳制技术，记录成年 Wistar 大鼠 DRG 神经元中 TTXr 钠电流。**结果**：Pue 在 0.01-2 mmol·L^{-1}浓度范围内，对 TTXr 钠电流的抑制率为 9.5 %-83.2 %。该抑制作用为浓度依赖性，可部分洗脱，但非频率依赖性或电压依赖性。Pue 不影响失活曲线，但使 1/2 最大激活电压由 -26 mV 升至 -16 mV，说明抑制了激活过程。**结论**：Pue 抑制大鼠 DRG 细胞中 TTXr 钠电流。