Puerarin blocks Na^+ current in rat ventricular myocytes[1]

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KEY WORDS puerarin; myocardium; patch-clamp techniques; sodium channels

ABSTRACT

AIM: To study the effect of puerarin (Pue) on Na^+ channel in rat ventricular myocytes. **METHODS:** Whole-cell patch-clamp technique was applied on isolated cardiomyocytes from rats. **RESULTS:** Pue inhibited cardiac I_{Na} in a positive rate-dependent and dose-dependent manner, with an IC_{50} of 349 μmol/L. The kinetics of blockage of cardiac sodium channel by Pue resembled the ClassIa/Ic of antiarrhythmic agents. Pue 300 μmol/L did not alter the shape of the *I-V* curve of I_{Na}, but markedly shifted the steady-state inactivation curve of I_{Na} towards more negative potential by 15.9 mV, and postponed the recovery of I_{Na} inactivation state from (21.9±1.6) ms to (54.4±3.4) ms ($P<0.01$). It demonstrated that the steady state of inactivation was affected by Pue significantly. **CONCLUSION:** Pue protected ventricular myocytes against cardiac damage and arrhythmias by inhibiting recovery from inactivation of cardiac Na^+ channels.

INTRODUCTION

Puerarin (Pue) prevents the heart from arrhythmias and improves myocardial reperfusion injury[1,2], and has been applied in the treatment of cardiovascular diseases in clinical settings. Recent findings showed that Pue blocked L-type calcium channel and K^+ channel in isolated guinea pig ventricular myocytes[3-5], inhibited the transient outward, and delayed rectified K^+ current in mouse hippocampal CA1 neurons[6].

The sodium channel in cardiomyocytes regulates the influx of I_{Na} which causes depolarization of the membrane, therefore, to affect voltage gated K^+ channels. Myocardial ischemia is likely to induce changes in the I_{Na} amplitude which affects the velocity of cardiac impulse conduction and the refractory period of cardiac excitability, leading to arrhythmogenesis. So suppression of the cardiac Na^+ channels is always regarded as an important indication for development of agents to potentially eliminate cardiac arrhythmias under clinical conditions. The suppressive effect of Pue on Na^+ channels was reported in neurons[7]. However, it is well known that the gene encoding and molecular construction of the Na^+ channels are different in the neural and cardiac tissue[8,9]. So it is interesting to observe that the mode of Pue on the cardiac Na^+ channel in order to understand mechanisms of Pue action on the heart.

MATERIALS AND METHODS

Isolation of cardiac myocytes Single ventricular myocytes were isolated from the hearts of adult Sprague-Dawley rats (200−250 g, ♀♂, Grade II, Certificate No 0003, the Experimental Animal Center of Beijing) as previously described[7]. Briefly, rats were anesthetized with pentobarbital sodium (30 mg/kg, ip).

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Hearts were rapidly excised and retrogradely perfused on a Langendorff apparatus, with a Ca^{2+}-free Tyrode's solution of the following composition (mmol/L): NaCl 137, KCl 5.4, NaH_2PO_4 1.2, $MgSO_4$ 1.2, Hepes 10, glucose 10, pH 7.4 for 5 min, then the perfusate was switched to Ca^{2+}-free Tyrode's solution containing collagenase B 0.5 g/L (Boehringer Mannheim Corp), protease XIV 0.2 g/L (Sigma Chemical Co), and bovine serum albumin (BSA) 1 g/L. The perfusate was oxygenated (95 % O_2+5 % CO_2) and maintained at 37 ºC. The ventricles were removed, cut into small chunks, and gently shaken in enzymatic solution. The cells were filtered through nylon mesh and washed twice with KB solution by centrifugation. Finally, the cells were stored in KB solution containing (mmol/L): KOH 70, KCl 40, KH_2PO_4 20, glutamic acid 50, $MgCl_2$ 3, taurine 20, egtazic acid 0.5, Hepes 10, glucose 10, pH 7.4.

Electrical recordings Dissected cells were transferred to a 0.5-mL chamber mounted on the plate of an inverted microscope (Nikon 810185, Japan), and perfused with normal Tyrode's solution at 2 mL/min. The whole cell patch-clamp technique was used to record membrane currents. Patch pipettes with a resistance of 1-3 MΩ, were pulled by a two-step puller (Narishige PP-93, Japan), and filled with an internal solution containing (mmol/L): CsF 125, CsCl 20, $NaCl_2$ 10, egtazic acid 5, Hepes 5, pH 7.2. Membrane currents were filtered by the amplifier at 3 kHz, sampled at 5 kHz and stored in a PC 486 computer using the Labmaster TL-1 interface (Axon Ins, USA). Current and voltage protocol generation and data acquisition and analysis were performed by the pClamp software (Ver 6.01, Axon Ins, USA).

To measure whole cell I_{Na}, myocytes were perfused with an external solution consisting of (mmol/L) choline chloride 100, $NaCl_2$ 20, CsCl 5, 4-AP 3, $MgCl_2$ 1.2, $CaCl_2$ 1.8, glucose 5.6, Hepes 5, $CoCl_2$ 3, TEA 20, pH 7.4. All experiments were carried out at room temperature (23 ºC to 25 ºC). I_{Na} amplitude was measured as the peak inward current with reference to the current at the end of the test pulse.

Chemicals Pue, a gift from Department of Phytochemistry, China Pharmaceutical University, was dissolved in extracellular solutions. The 4-AP (4-aminopyridine), CsCl, CsF, TEA, $CoCl_2$, egtazic acid, and BSA were purchased from Sigma Chemical Co.

Data analysis All data were analyzed by pCLAMP 6.0 procedures (Axon Ins, USA) and Sigmaplot (Jandel Scientific) software. All values were expressed as mean±SD. Statistical significance was analyzed by paired or unpaired *t*-test

RESULTS

Concentration-dependent inhibition of cardiac I_{Na} by Pue I_{Na} was elicited by a 30-ms pulse to -40 mV from the holding potential at -90 mV. TTX 30 μmol/L completely blocked the current to confirm that the current was cardiac I_{Na} (n=5, not shown). Effect of Pue reached a steady state around 8-10 min. So I_{Na} was recorded after application of Pue for 10 min. The suppressive effect of Pue on the cardiac I_{Na} currents was concentration-dependent (Fig 1) in the range of 10-1000 μmol/L with the IC_{50} value of 349 μmol/L and the maximal suppression was up to 68.4 %±15.8 % by Pue 1000 μmol/L (n=7). After washout of Pue, the cardiac I_{Na} recovered substantially and the effect of Pue was reversible in drug-free solution (n=7 , Fig 2A).

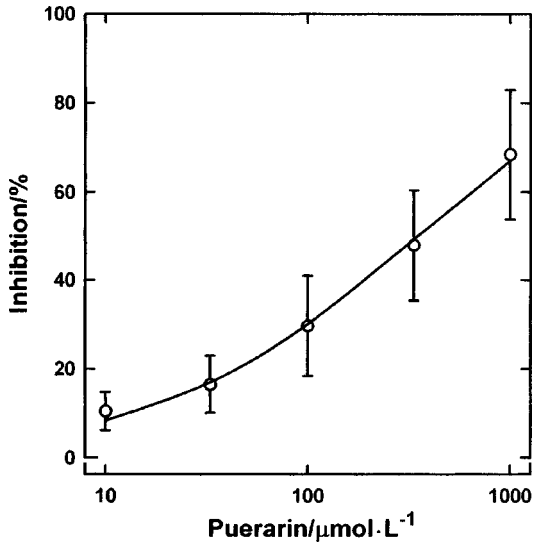

Fig 1. Concentration-dependent effect of Pue on I_{Na} in rat ventricular myocytes. n=7 cells. Mean±SD.

Effects of Pue on current-voltage relationship (I-V) of cardiac I_{Na} The I-V relationship of I_{Na} was shown in Fig 2B. Current traces were elicited by 30-ms depolarizing pulses to potentials ranging from -80 mV to +40 mV in 10-mV increments at 0.5 Hz, when the holding potential was -90 mV. Before the application of drug, the current began to be activated at -60 mV, reaching maximum amplitude near -40 mV. The reversal potential was (36.0±0.7) mV. Pue 300 μmol/L suppressed the cardiac I_{Na}, without modifying the maximum activation potential and the reversal potential

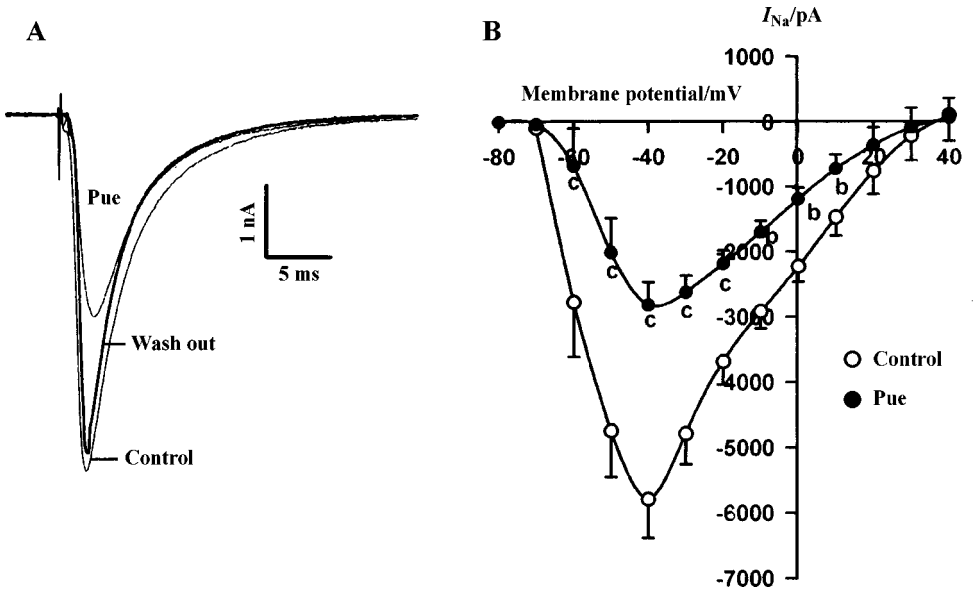

Fig 2. Effect of Pue 300 μmol/L on *I-V* relationship of I_{Na} in rat ventricular myocytes. A) Current traces obtained in the absence and presence of the drug. B) *I-V* relationship of I_{Na}. *n*=9 cells. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control.

[(37.1±1.3) mV]. The peak of I_{Na} was decreased by 45 %±5 % at -40 mV and the shape of *I-V* curve was not altered by Pue ($P>0.05$, $n=9$).

Effects of Pue on activation and inactivation kinetics of cardiac I_{Na} On the basis of data obtained from *I-V* relationship, the activation curves of cardiac I_{Na} were determined before and after application of Pue 300 μmol/L. The activation curve was fitted by the Boltzmann equation $G/G_{max}=1/[1+\exp(V_h-V)/k]$, where G is the membrane conductance at potential V, V_h is the half activation voltage, k is a slope factor. The V_h measured before and after the application of Pue was (-52.2±2.6) mV and (-57±3) mV ($P>0.05$), respectively, with k value as (4.8±0.7) mV and (5.1±0.9) mV, separately ($P>0.05$, $n=9$). Pue slightly shifted the steady-state activation curve of cardiac I_{Na} towards more positive potential by 4.9 mV, but did not obviously affect the activation characteristics of I_{Na}.

The steady-state inactivation curves of I_{Na} were obtained by use of a double-pulse protocol, a 3-s condition prepulse of various potentials (from -120 mV to +30 mV, holding potential at -90 mV) was followed by a 30-ms test pulse to -40 mV. The inactivation curve was also fitted by the Boltzmann equation $I/I_{max}=1/[1+\exp(V-V_h)/k]$, where V_h is the half inactivation voltage, k is a slope factor. The V_h and k of the cardiac I_{Na} were (-67±3) mV and (8.3±1.2) mV in control; and (-82±4) mV ($P<0.01$) and (13.7±1.5) mV ($P<0.01$), in the presence of Pue 300 μmol/L ($n=9$), respectively. The steady-state inactivation curve of I_{Na} was obviously shifted towards more negative potential by 15.9 mV in the presence of Pue 300 μmol/L (Fig 3).

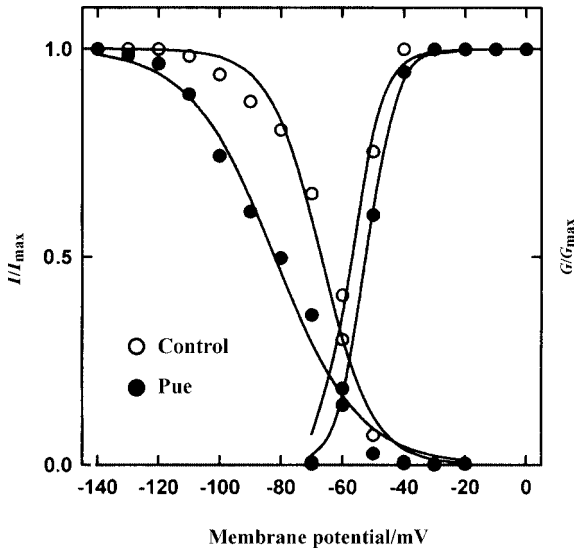

Fig 3. Effect of Pue 300 μmol/L on activation and inactivation kinetics of I_{Na} in rat ventricular myocytes. *n*=9 cells.

Effects of Pue on the recovery kinetics of cardiac I_{Na} The time course of recovery of I_{Na} from steady state inactivation was studied using a conventional double-pulse protocol. A 3-s pre-pulse to -40 mV from holding potential of -90 mV was followed by various recovery durations and then by a test pulse to -40 mV for 50 ms. The recovery process of cardiac I_{Na} could be fitted by a single exponent and the time constant of

recovery from the steady state inactivation was (21.9± 1.6) ms and (54.4±3.4) ms in control and Pue 300 μmol/L group (P<0.01, n=9), respectively. It was confirmed that Pue delayed the recovery time constant of cardiac I_{Na} from inactivation state (Fig 4).

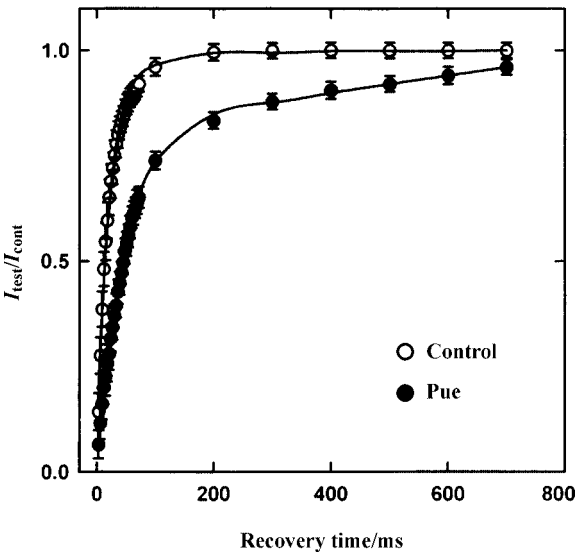

Fig 4. Effect of Pue 300 μmol/L on the recovery curve of I_{Na} from inactivation in rat ventricular myocytes. n=9 cells. Mean±SD.

Positive rate-dependent effect of Pue on cardiac I_{Na} The rate-dependent effect of Pue 300 μmol/L was studied by a series of 30 depolarizing pulses (30 ms duration) from a holding potential of -90 mV to -40 mV at different stimulation frequencies (1, 2, and 4 Hz) (n=8). The suppression of the sodium channel was enhanced along with an increase in the frequency of stimuli showing a positive rate-dependence. The kinetic of positive rate-dependence was rapidly onset but it was very slow approaching to a plateau (Fig 5).

DISCUSSION

Pue inhibited cardiac I_{Na} in a concentration-dependent and positive rate-dependent manner . The positive rate-dependence developed rapidly but reached the plateau slowly in a manner resembling the characteristics of Class Ia/Ic antiarrhythmic agents[11].

The blocking effect of Pue seems to be more sensitive in cardiac myocytes than in the neuron[7]. The potency of the inhibition of sodium channels by Pue is 3 fold more potent in cardiac myocytes than that in neuron, IC_{50} 349 *vs* 934 μmol/L[7]. The difference of sodium channels in neuronal and cardiac tissue has been reported[8,9], concentrations of TTX to suppress the neuronal sodium channels are in the range of nmol/L but as high as 1-10 μmol/L to suppress the cardiac I_{Na}[9]. The difference in the pharmacological action stemmed from differentiated coding by genes. Subunits of sodium channels, one α subunit and two β (β1 & β2) subunits, are instituted in a large family which consists of some isoforms in the brain, heart, and skeletal muscle. The cardiac α subunit in possession of 4 domains of channels is encoded by gene SCN_5A, which is located at chromosome 3 p14, totally different from the gene that encodes neuronal sodium channels (Tab 1). There are three isoforms of neuronal Na^+ channels, the SCN_1A, SCN_2A, and SCN_3A[12]. Different genes contribute to different structure of the cardiac α-subunit of which there is systeine at position 373 in the amino sequence against phenylalanine/tyrosine in the neurons resulting in the divorced responses to TTX (Tab 1).

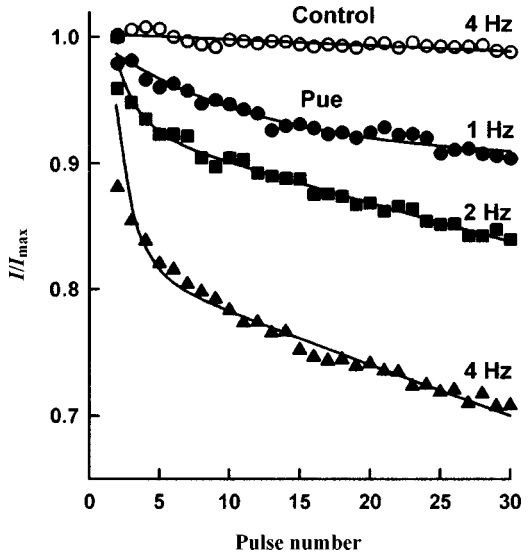

Fig 5. Positive frequency-dependent effect of Pue 300 μmol/L on the I_{Na} in rat ventricular myocytes. n=8 cells.

The inactivation curve of the cardiac I_{Na} was shifted by Pue toward more negative potential but it was not affected in the neurons. Furthermore the time constant of recovery from inactivation was markedly increased in the cardiac sodium channels but not in the neurons[7].

Cardiac injury caused by myocardial ischemia or hypoxia induces the accumulation of cytosol Na^+ which leads to an overload of Ca^{2+} by activation of Na^+-Ca^{2+} exchanger[12]. During hypoxia/ischemia, intracellular acidosis is commonly observed due to activation of Na^+-H^+ exchange via a rise in cytosolic Na^+ concentration[13]. Na^+-H^+ exchange and Na^+ channels are considered as the two main sources for developing intracellular Na^+

Tab 1. Comparison of the α subunit of sodium channel in the brain and heart.

Gene	Chromosome	α-Subunit	Mutation causing diseases
Cardiac			
SCN_5A	3p21	TTX insensitive isoform (1-10 μmol/L): position 383: cysteine	LQTS (Long QT syndrome) severe cardiac arrhythmias and cardiac sudden death
Brain			
SCN_1A (SCN_2A,	2q21-q33 SCN_3A)	TTX sensitive isoform (nmol/L): position 383: phenylalanine/tyrosine.	epilepsy

accumulation under pathological processes[12,13]. The effectiveness of Na^+-channel blockers in cardioprotection, such as limiting post-ischemic arrhythmias and improving mechanical recovery, is due to inhibition of Na^+-H^+ exchange, Na^+ channel, or Na^+/Ca^{2+} exchange[14-16].

The blocking effect of Pue on the sodium channels is not selective. Because Pue also blocked Ca^{2+} and K^+ channels[4-6]. This phenomena of non-selective effect on ion channels could also be seen in other compounds isolated from plant origin: berberine, palmatine, and the derivative CPU 86017[17,18]. In clinical practice Pue has been applied to treat myocardial ischemia. These findings suggest that Pue can block the inactivation state of Na^+ channel and delay recovery of Na^+ channel from inactivation state to inhibit the cardiac arrhythmias

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