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Original Article

Action potential bursts in central snail neurons elicited by paeonol: roles of ionic currents

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Aim: To investigate the effects of 2′-hydroxy-4′-methoxyacetophenone (paeonol) on the electrophysiological behavior of a central neuron (right parietal 4; RP4) of the giant African snail (Achatina fulica Ferussac).
Methods: Intracellular recordings and the two-electrode voltage clamp method were used to study the effects of paeonol on the RP4 neuron.
Results: The RP4 neuron generated spontaneous action potentials. Bath application of paeonol at a concentration of ≥500 μmol/L reversibly elicited action potential bursts in a concentration-dependent manner. Immersing the neurons in Co^{2+}-substituted Ca^{2+}-free solution did not block paeonol-elicited bursting. Pretreatment with the protein kinase A (PKA) inhibitor KT-5720 or the protein kinase C (PKC) inhibitor Ro 31-8220 did not affect the action potential bursts. Voltage-clamp studies revealed that paeonol at a concentration of 500 μmol/L had no remarkable effects on the total inward currents, whereas paeonol decreased the delayed rectifying K^+ current (I_{KD}) and the fast-inactivating K^+ current (I_A). Application of 4-aminopyridine (4-AP 5 mmol/L), an inhibitor of I_A, or charybdotoxin 250 nmol/L, an inhibitor of the Ca^{2+}-activated K^+ current ($I_{K(Ca)}$), failed to elicit action potential bursts, whereas tetraethylammonium chloride (TEA 50 mmol/L), an I_{KD} blocker, successfully elicited action potential bursts. At a lower concentration of 5 mmol/L, TEA facilitated the induction of action potential bursts elicited by paeonol.
Conclusion: Paeonol elicited a bursting firing pattern of action potentials in the RP4 neuron and this activity relates closely to the inhibitory effects of paeonol on the I_{KD}.

Keywords: paeonol; neuron; snail; action potential bursts; fast-inactivating K^+ current; delayed rectifying current

Introduction

The Chinese have used herbs to treat a wide variety of medical ailments for several thousand years. Moutan cortex, the root bark of *Paeonia suffruticosa* Andrews, has long been used for its antipyretic and anti-inflammatory effects in traditional Chinese medicine[1, 2]. One of the major components of Moutan cortex is 2′-hydroxy-4′-methoxyacetophenone (paeonol), which has been reported to possess analgesic, antipyretic and antibacterial properties as well as anti-inflammatory and antioxidant activities and an ability to suppress ADP or collagen-induced human blood platelet aggregation[1, 3, 4].

Recent pharmacological experiments have shown that paeonol protects against reperfusion-induced myocardial damage[5]. Paeonol has also been reported to block the L-type calcium current in cardiac myocytes, thereby decreasing the excitability of cardiac tissue[6]. In a recent study carried out in guinea pig ventricular myocytes using patch-clamp techniques, paeonol decreased the action potential upstroke phase, an action associated with the blockade of the voltage-gated fast sodium channel[7].

Other research indicates that paeonol has neuroprotective effects. For example, paeonol has been shown to protect rat neurons from oxygen-glucose deprivation-induced injury by alleviating morphological damage and increasing neuron viability[8]. Paeonol has ameliorated neuronal damage in both the hippocampus and temporal cortex in D-galactose-treated

mice[9]. These results suggest that paeonol possesses anti-aging properties and may have potential in the treatment of neurodegenerative diseases. However, scant evidence exists as to the effects of paeonol on neuronal excitability.

The central nervous system of the gastropod snail contains large, identifiable neurons with known pharmacological profiles and synaptic inputs. The size, accessibility and relative simplicity of the molluscan neuronal network makes it an ideal *in vitro* preparation for electrophysiological and neuropharmacological studies[10-12]. Identifiable neurons in the ganglia can undergo repeated investigations into drug-related effects on the same neuron. Snail ganglia contain many identifiable neurotransmitters and receptors, and their neurons are used for biological studies[11, 13, 14]. In our previous studies, CNS stimulants, including d-amphetamine, cocaine and methamphetamine, elicited *in vitro* action potential bursts in the central right parietal 4 (RP4) neuron of the African snail *Achatina fulica* Ferussac[13, 15-17].

Few studies have examined the effects of paeonol on neuronal excitability. The present study aimed to determine the effects of paeonol on membrane potentials and ionic currents in the central RP4 neuron using the conventional two-electrode voltage clamp technique.

Materials and methods

Experiments were performed on identified central RP4 neurons from the subesophageal ganglia of the African snail *Achatina fulica* Ferussac. The ganglia were pinned to a Sylgard-coated perfusion chamber base (volume=2 mL) and removed from the connective tissue sheath to allow easy identification and penetration by microelectrodes[13, 16, 17].

Intracellular recordings were made with a Gene clamp 500 amplifier (Axon Instruments, Foster City, CA, USA). Membrane potentials were recorded with microelectrodes (5–6 MΩ) filled with 3 mol/L potassium chloride (KCl). The experimental chamber was perfused with the control solution, *ie*, (in mmol/L) NaCl (85), KCl (4), CaCl$_2$ (8), MgCl$_2$ (7), and Tris-HCl (10) at pH 7.6, at a room temperature of 23–24 °C with a perfusion speed of 8 mL/min. Neurons were studied only if the negativity of their resting membrane potentials (RMPs) exceeded -50 mV, the time constant was around 5–8 ms and the rate of rise of the action potentials was around 5–8 V/s[18].

The ionic currents of the central snail neurons were measured using the two-electrode voltage clamp method. Two microelectrodes were inserted into the neuron for current injection and voltage clamp studies. The recording electrode (5–6 MΩ) and current injection electrode (1–5 MΩ) were filled with 3 mol/L KCl. All potentials and currents were recorded on tape via a digitalizing unit (Digidata 1440, Axon Instruments) and analyzed using pCLAMP software.

Na$^+$-free solution was used to measure the fast-inactivating K$^+$ current (I_A). The currents were elicited by 200 ms test potentials of -60, -50, -40, and -30 mV from a holding potential of -100 mV[19]. A P/4 leak subtraction subpulse routine supplied by the pCLAMP software was used to measure the I_A.

For measuring the I_{KD}, Na$^+$-free and Co^{2+}-substituted Ca^{2+}-free solutions were used to remove Ca^{2+} currents (I_{Ca}), Na$^+$ currents (I_{Na}) and the Ca^{2+}-activated K$^+$ current ($I_{K(Ca)}$). RP4 neuron currents were elicited with 500-ms-long voltage clamp steps from a holding potential of -60 mV to test potentials between -70 and +50 mV at intervals of 10 mV. The potential recording and current injection electrodes were filled with 3 mol/L KCl to measure both types of K$^+$ current.

The ganglia were perfused with the following solutions: (a) physiological solution; (b) Na$^+$-free solution: equimolar amounts (85 mmol/L) of Tris were added to replace Na$^+$ ions; (c) Co^{2+}-substituted Ca^{2+}-free solution: equimolar amounts (8 mmol/L) of Co^{2+} were added to replace Ca^{2+} ions; and (d) Na$^+$-free and Co^{2+}-substituted Ca^{2+}-free solution: equimolar amounts of Tris (85 mmol/L) and Co^{2+} (8 mmol/L) were added to replace Na$^+$ and Ca^{2+} ions, respectively. The tetraethylammonium chloride (TEA)-containing solution was prepared by replacing equimolar quantities of NaCl or Tris-Cl with TEA[20].

Paeonol, 4-aminopyridine (4-AP), TEA, KT-5720, Ro 31-8220 and charybdotoxin were purchased from the Sigma Chemical Company (St Louis, MO, USA). All drug stocks were made with double-distilled water, except for KT-5720 and Ro 31-8220, which were prepared in dimethyl sulfoxide (DMSO), and paeonol, which was prepared in ethanol. The presence of DMSO (≤0.1%) and ethanol (≤1%) did not affect the RMPs, amplitude or frequency of the spontaneous firing of action potentials in the RP4 neuron.

Tests of significance regarding any differences between the amplitude and frequency of the action potentials, RMPs and currents post-treatment compared with the pre-drug controls were determined by the Student's paired *t*-test. Differences were considered significant at *P*<0.05.

Results

Effects of extracellular application of paeonol on spontaneous action potentials of the RP4 neuron

The RMP, amplitude and frequency of spontaneously generated action potentials of the identified RP4 neuron are shown in Table 1. The electrical characteristics of the RP4 neuron were similar to those detailed in a previous report[15].

The effects of paeonol (150 µmol/L, 500 µmol/L, and 1.5 mmol/L) on the spontaneous firing action potentials of the RP4 neuron are shown in Figure 1 and Table 1. At a concentration of 150 µmol/L, paeonol did not alter the action potential firing pattern. As shown in Table 1, at 20 min after extracellular perfusion of paeonol (150 µmol/L), the frequency of spontaneously firing action potentials and the RMP remained unchanged. No action potential bursts were observed even after 1 h of incubation. However, higher concentrations of paeonol did change the action potential firing pattern. Twenty minutes after extracellular perfusion of paeonol (500 µmol/L), the firing pattern changed from regularly spaced single spikes to one in which bursts of between 2 and 10 action potentials were separated by large hyperpolarizations of the membrane potentials (up to 9 mV) that lasted for 5–15 s each. The RMP, action potential amplitude and bursting frequency and the

Table 1. Effects of paeonol, Ro 31-8220, KT-5720, Co^{2+}-substituted Ca^{2+}-free solution, 4-AP, TEA, and charybdotoxin on the resting membrane potential, amplitude and frequency of spontaneously generated action potentials of RP4 neurons, and effects of KT-5720, Ro 31-8220, Co^{2+}-substituted Ca^{2+}-free solution and TEA on paeonol-elicited action potential bursts. Values are expressed as the mean±SEM. (*n* being the number of neurons tested). $^{b}P<0.05$ *vs* paeonol (500 µmol/L)-elicited action potential bursts. $^{e}P<0.05$ *vs* the data in physiological solution (control).

Variable		*n*	RMP (mV)	Amplitude of action potential (mV)	Frequency of single spikes (pulse/min)	Frequency of bursts (burst/min)	Number of action potential/ burst
Paeonol	Control	7	-57.7±1.8	85.1±3.3	50.1±11.2		
	Paeonol (150 µmol/L)	4	-58.5±2.7	86.1±6.9	47.3±11.8		
	Paeonol (500 µmol/L)	6	-55.1±1.4	80.8±5.1		8.1±1.6	5.0±0.6
	Paeonol (1.5 mmol/L)	3	-59.3±2.3	83.3±9.6		5.0±1.5	8.7±1.7^{b}
Ro 31-8220+Paeonol	Control	5	-57.0±1.1	96.9±2.1	28.5±5.0		
	Ro 31-8220 (20 µmol/L)	4	-57.7±1.2	98.8±2.2	30.0±6.7		
	Ro 31-8220 (10 µmol/L)+ Paeonol (500 µmol/L)	3	-54.0±2.3	100.3±4.5		7.0±1.0	4.5±2.5
KT-5720+Paeonol	Control	3	-63.0±0.6	96.1±3.7	25.3±3.3		
	KT-5720 (10 µmol/L)	3	-61.3±1.2	97.8±4.3	30.7±3.8		
	KT-5720 (10 µmol/L)+ Paeonol (500 µmol/L)	3	-61.7±3.5	96.1±7.3		7.0±1.5	4.3±0.3
Paeonol in Ca^{2+}-free	Control	6	-63.0±2.1	98.2±3.2	26.2±4.9		
	Ca^{2+}-free	3	-49.8±1.3^{b}	65.7±3.5^{e}	22.7±5.4		
	Paeonol (500 µmol/L) in Ca^{2+}-free	3	-58.3±1.5	73.5±5.3^{e}		2.7±1.2^{e}	17.3±5.3^{e}
Paeonol+TEA	Control	3	-59.3±3.3	82.3±5.2	51.3±5.3		
	TEA (5 mmol/L)	4	-57.5±1.6	86.0±3.2	29.5±3.0^{e}		
	Paeonol (150 µmol/L)+ TEA (5 mmol/L)	3	-56.3±0.7	92.7±5.6		14.7±6.7	3.7±0.9
4-AP	Control	4	-55.3±2.8	90.2±6.0	48.8±16.4		
	4-AP (5 mmol/L)	4	-56.3±4.2	91.5±6.1	49.0±14.8		
TEA	Control	4	-60.5±1.0	79.9±3.2	23.5±9.2		
	TEA (50 mmol/L)	4	-58.0±4.0	84.4±5.6		10.5±5.5	3.1±0.7
Charybdotoxin	Control	3	-59.0±1.7	87.3±2.6	49.6±6.0		
	Charybdotoxin (250 nmol/L)	3	-57.6±1.2	88.7±3.2	39.3±1.5		

number of action potentials for each burst are shown in Table 1. These effects of paeonol continued throughout its application (for up to 3 h).

At the highest concentration of 1.5 mmol/L, paeonol enhanced the pattern of action potential bursts. The membrane potential underwent a phasic depolarization followed by a sustained depolarization. The number of action potentials for each burst was increased compared with those for the bursts elicited by paeonol 500 µmol/L (Table 1 and Figure 1). After 30 min of continuous washing, the spontaneously generated spikes of the central neuron returned to control levels.

Effects of Co^{2+}-substituted Ca^{2+}-free solution on paeonol-elicited potential changes of the RP4 neuron

To test the effects of Co^{2+}-substituted Ca^{2+}-free solution on paeonol-elicited changes in action potential bursts, the RP4 neuron underwent 30 min of treatment with Co^{2+}-substituted Ca^{2+}-free solution alone (Figure 2A2) or in combination with paeonol (Figure 2B2). Co^{2+}-substituted Ca^{2+}-free solution decreased both the RMP and the amplitude of action potentials (Table 1). No action potential bursts were elicited (Figure 2A2).

In contrast, perfusion with the Co^{2+}-substituted Ca^{2+}-free solution containing paeonol (500 µmol/L) elicited action potential bursts (Figure 2B2 and Table 1). The RMP and the amplitude of action potentials were decreased, as shown in Table 1. The number of action potentials for each burst was higher, and the frequency of bursts lower, compared with the
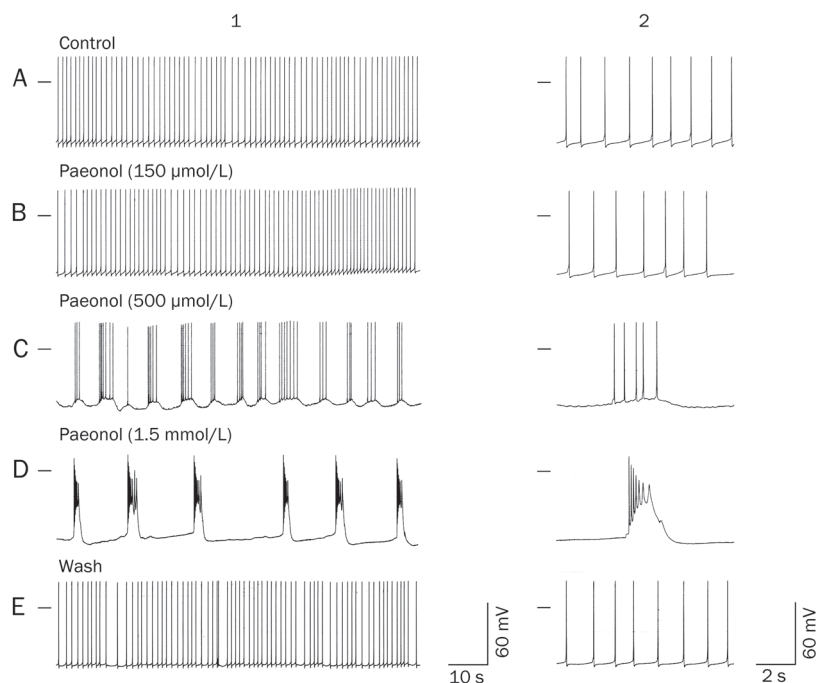
Figure 1. Effects of paeonol on the RP4 neuron. A, B, C, D, and E were recorded from the same neuron. A1: Control; the neuron shows spontaneous firing of action potentials. B1, C1, and D1: Membrane potentials recorded 20 min after the administration of paeonol (150, 500 and 1500 μmol/L), respectively. E1: Membrane potentials measured at 30 min after the washing off of paeonol from D1. A2, B2, C2, D2, and E2: Expanded pictures showing individual action potentials related to A1, B1, C1, D1, and E1, respectively. The horizontal bar on the upper left side indicates the membrane potential at 0 mV. Notably, action potential bursts were not elicited by paeonol at 150 μmol/L but were reversibly elicited at concentrations of 500 μmol/L and 1.5 mmol/L.
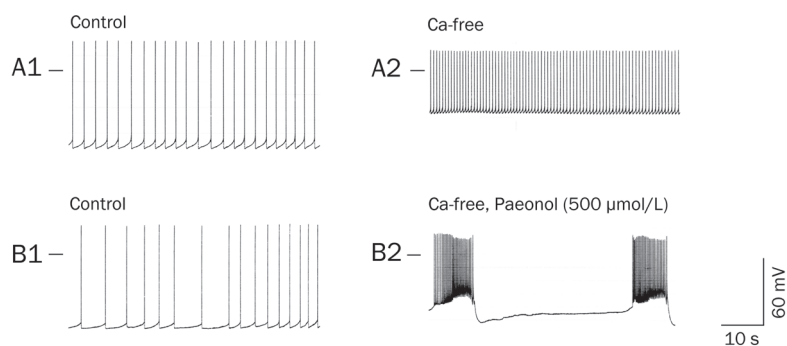
Figure 2. Effects of Co^{2+}-substituted Ca^{2+}-free solution on paeonol (500 μmol/L)-elicited action potential bursts in the RP4 neuron. A1 and A2 were recorded from one neuron, while B1 and B2 were recorded from another. A1: Spontaneous action potentials recorded from an RP4 neuron in normal physiological solution. A2: The potentials at 30 min after perfusion with Co^{2+}-substituted Ca^{2+}-free solution. B1: Spontaneous action potentials recorded from an RP4 neuron in normal physiological solution. B2: Membrane potentials recorded at 20 min after perfusion with Co^{2+}-substituted Ca^{2+}-free solution containing paeonol (500 μmol/L).

bursting elicited by paeonol at 500 μmol/L in normal physiological solution.

The removal of extracellular calcium ions did not abolish the paeonol (500 μmol/L)-induced bursting pattern of action potentials, but it reduced the action potential amplitudes.

Effects of a PKA inhibitor on paeonol-elicited potential changes of the RP4 neuron

We then sought to determine whether the cAMP-protein kinase A (PKA) signaling pathway is implicated in the generation of paeonol-elicited action potential bursts. As shown in Table 1 and Figure 3B, after 40 min of incubation with the PKA inhibitor KT-5720 (10 μmol/L)[21], the frequency of the spontaneous action potentials, the RMP and the amplitude of the action potentials did not differ from those of the control RP4 neuron.

As mentioned earlier, paeonol (500 μmol/L) elicited action potential bursts in the RP4 neuron. When KT-5720 10 μmol/L-pretreated RP4 neurons were further incubated with

paeonol (500 μmol/L) for 20 min, action potential bursts were elicited, as shown in Table 1 and Figure 3C.

Pretreatment with KT-5720 failed to prevent paeonol-elicited action potential bursts.

Effects of a PKC inhibitor on paeonol-elicited potential changes of the RP4 neuron

To determine whether protein kinase C (PKC) is involved in the generation of action potential bursts elicited by paeonol, we tested the effects of PKC inhibition with Ro 31-8220 (20 μmol/L)[22]. After a 40-min incubation, the RMP, amplitude and frequency of single spikes in the RP4 neuron were not significantly different from values obtained from neurons in normal physiological solution (Table 1 and Figure 3G). Further incubation of Ro 31-8220-pretreated RP4 neurons with paeonol (500 μmol/L) for 20 min resulted in action potential bursts (Table 1 and Figure 3H).

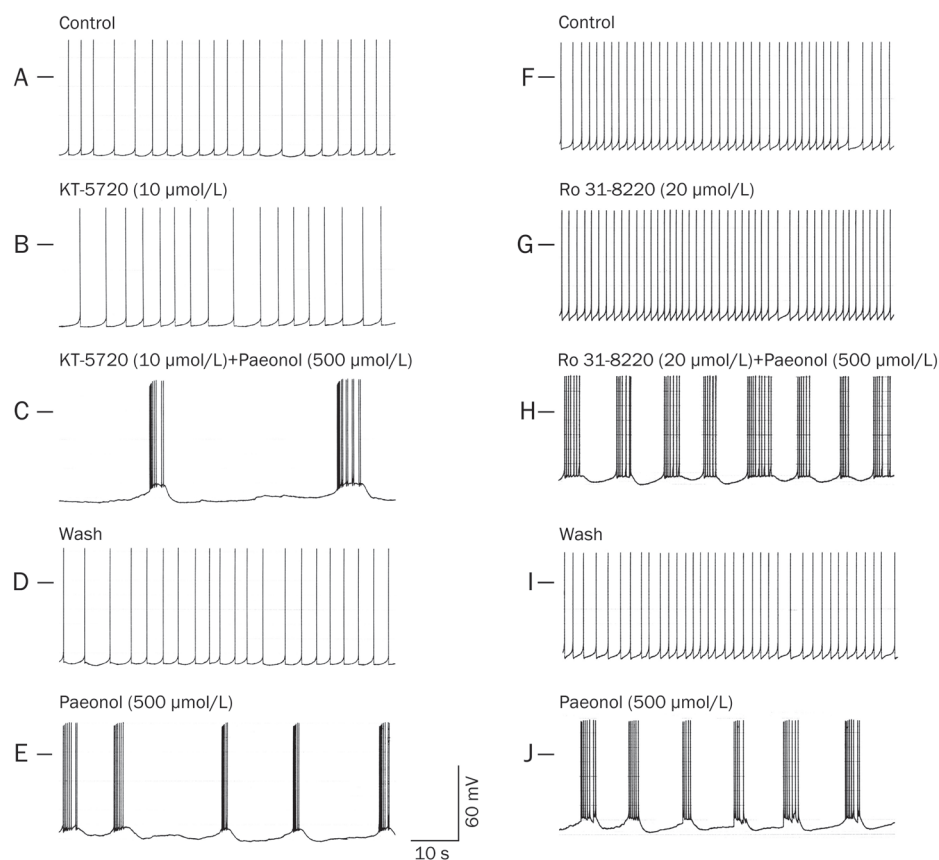Pretreatment with Ro 31-8220 failed to abolish paeonol-generated action potential bursts.

Figure 3. Effects of KT-5720 (10 µmol/L; A–E) and Ro 31-8220 (20 µmol/L; F–J) on paeonol-elicited action potential bursts in the RP4 neuron. A, B, C, D, and E were recorded from the same neuron, while F, G, H, I, and J were recorded from another. A and F: Controls; spontaneous action potentials. B and G: Potentials at 40 min after perfusion with KT-5720 (10 µmol/L) and Ro 31-8220 (20 µmol/L), respectively. C and H: Potentials at 20 min after further incubation of the neuron with paeonol (500 µmol/L) plus KT-5720 (10 µmol/L) and paeonol (500 µmol/L) plus Ro 31-8220 (20 µmol/L), respectively. D and I: Potentials at 30 min after washing from C and H, respectively. E and J: Potentials at 20 min after incubation of the neuron with paeonol (500 µmol/L) from D and I, respectively. The horizontal bar on the upper left side indicates the membrane potential at 0 mV. Note that paeonol elicited action potential bursts in the presence of KT-5720 (10 µmol/L) and also Ro 31-8220 (20 µmol/L).

Effects of paeonol on the fast ionic currents of the RP4 neuron

The fast ionic currents of the RP4 neuron clamped at 70 ms durations are shown in Figure 4. The membrane potentials were held at -60 mV and stepped to the testing potentials of -50, -40, -30, -20, -10, 0, 10, 20, and 30 mV in 70-ms-long durations. The total inward currents observed in various voltage clamping command steps are shown in Figure 4A. The relationships between the peak inward currents and the test potentials are shown in Figure 4B. The inward current was obvious when the potential was stepped to positive levels that exceeded -40 mV. The maximum peak inward current was observed after voltage stepping to test potentials between -20 mV and -10 mV. As shown in our previous study, the removal of either extracellular Na^+ ions or Ca^{2+} ions decreased the amplitude of the total inward currents[13, 23]. Both the I_{Ca} and the I_{Na} contributed to the total inward currents of the RP4 neuron. As shown in Figure 4B, paeonol (500 µmol/L) did not significantly decrease the fast inward currents in a series of voltage steps.

Effects of paeonol on the I_A current

Measurement of the I_A followed Thompson's method[19]. Currents were elicited by 200 ms test potentials of -60 mV, -50, -40, and -30 mV from a holding potential of -100 mV. An example of the I_A current is shown in Figure 5; the I_A current was completely abolished if 4-AP was applied to the Na^+-free solution for 20 min (Figure 5F).

As shown in Figure 5, perfusion of neurons for 20 min with paeonol (150 µmol/L) did not affect the peak amplitude of the I_A. However, the I_A was decreased after a 20 min perfusion with paeonol at higher concentrations of 500 µmol/L and also 1.5 mmol/L (by 40.0%±6.7%, $n=4$ and 85.7%±0.6%, $n=3$, respectively; at a test potential of -30 mV). Paeonol 150 µmol/L had no effect on the I_A, whereas higher concentrations of paeonol (500 µmol/L and 1.5 mmol/L) successfully decreased the I_A.

Effects of paeonol on the steady-state outward K^+ current of the RP4 neuron

The steady-state outward current of the RP4 neuron was measured by 500-ms-long voltage clamp steps in Na^+-free and Co^{2+}-substituted Ca^{2+}-free solution (Figure 6A). In this condition, the I_A and delayed rectifying K^+ (I_{KD}) components of the outward currents were identified by 4-AP and TEA, as in our previous study[13]. The amplitude of the outward current at 500 ms was significantly reduced (by 40.7%±3.1%, $n=3$; at a test potential of 50 mV) after perfusion with TEA (50 mmol/L) for 40 min (Figure 6B). If 4-AP (5 mmol/L) was applied to the bath solution for 20 min, the outward current remained unchanged. The sensitivity of the outward current to TEA indicates that this is a type of I_{KD}. However, there was a sustained residual TEA-insensitive current[13].

The effects of paeonol on the steady-state outward current in Na^+-free and Co^{2+}-substituted Ca^{2+}-free solution are shown in
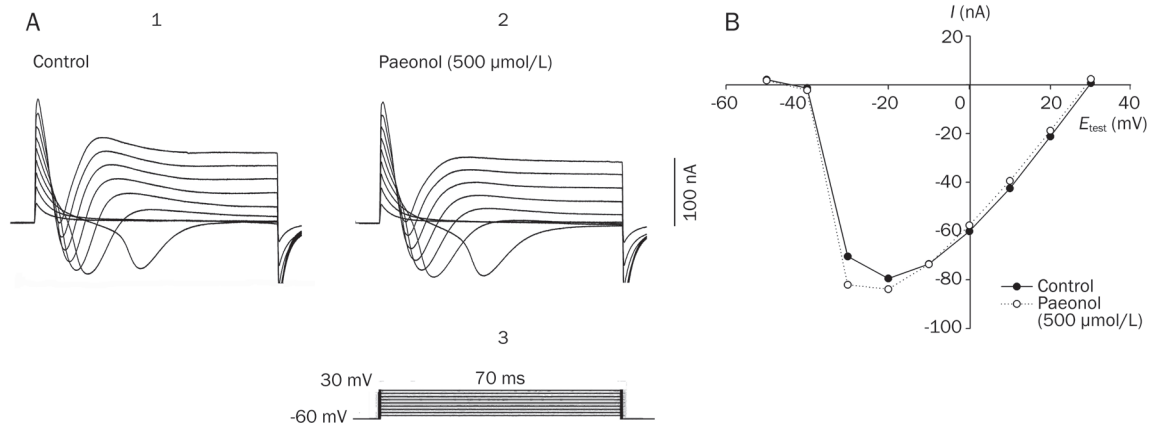
Figure 4. (A) Effects of paeonol on the total inward currents of the RP4 neuron. The membrane currents were elicited from a holding potential of -60 mV to test potentials of -50, -40, -30, -20, -10, 0, 10, 20, and 30 mV of 70-ms-long durations in normal physiological solution. A1: Control; total inward and outward currents recorded in normal physiological solution. A2: Total inward and outward currents recorded at 20 min after incubation with paeonol (500 µmol/L). A3: Voltage step commands. (B) Current-voltage relationships of the peak total inward currents before (●), and at 20 min after (○), paeonol (500 µmol/L) application. Note that paeonol (500 µmol/L) did not decrease the total inward currents.
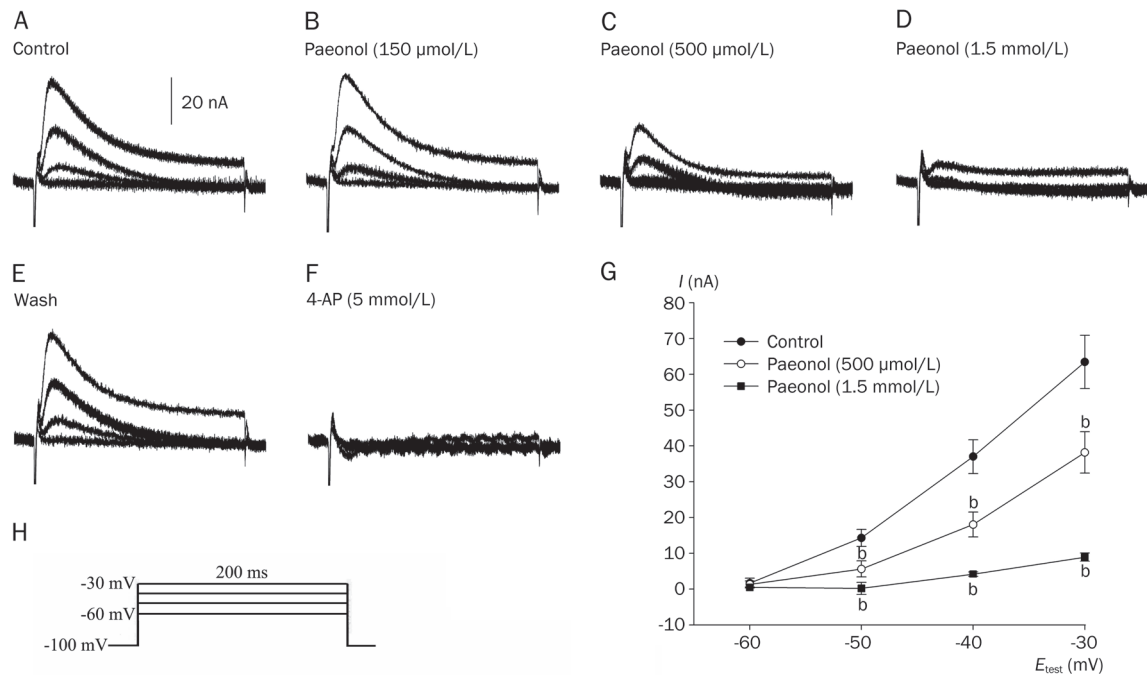

Figure 5. Effects of paeonol on the peak amplitude of the I_A current of the RP4 neuron. A: Control I_A currents recorded in Na$^+$-free solution. B, C, and D: I_A currents recorded at 20 min after paeonol (150 µmol/L), paeonol (500 µmol/L) and paeonol (1.5 mmol/L) administration, respectively. E: I_A currents recorded at 30 min after washing off from D. F: I_A currents recorded at 20 min after 4-AP (5 mmol/L) administration from E. G: Current-voltage relationships of the I_A currents before (●) and after paeonol administration at 500 µmol/L (○) and 1.5 mmol/L (■), respectively. H: Voltage step commands. Note that paeonol (500 µmol/L and 1.5 mmol/L) significantly decreased the I_A at 20 min after treatment ([b]$P<0.05$ *vs* control. $n=3-4$).

Figure 6A. The corresponding amplitudes of the steady-state outward currents, as measured at 500 ms of the outward current, and the steady-state current-voltage ($I-V$) relationships are shown in Figures 6C1 and 6C2. When neurons underwent 20 min of treatment with paeonol at 150 µmol/L, the steady-state outward current was not affected, whereas a 20-min

treatment with paeonol at 500 µmol/L significantly decreased the steady-state outward current when subjected to test potentials in the range of 0 mV to 50 mV. The current amplitude measured at 500 ms was reduced by 44.8±5.2 nA (by 18.2%±1.8% *vs* controls, $P<0.05$, $n=5$) at a test potential of 50 mV. A further 20-min treatment with paeonol at 1.5 mmol/L
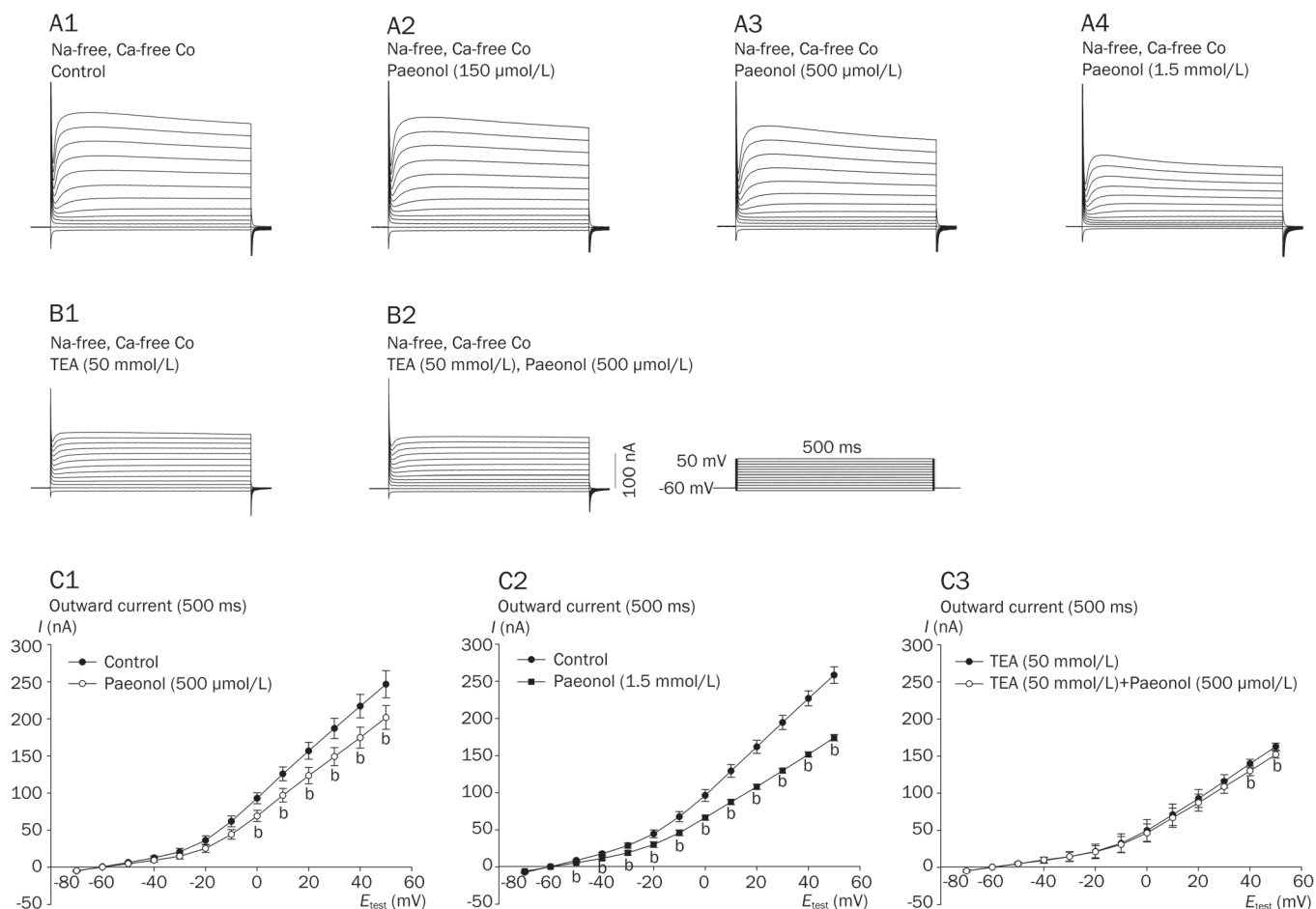
Figure 6. Effects of paeonol on steady-state outward currents of the RP4 neuron in Na$^+$-free and Co^{2+}-substituted Ca^{2+}-free saline in the absence and presence of TEA (50 mmol/L). A and B were recorded from two different RP4 neurons. Currents were elicited by 500-ms-long command steps from holding potentials of -60 mV to test potentials (*ie*, from -70 to 50 mV at intervals of 10 mV). A1: Control; steady-state outward currents of the RP4 neuron in Na$^+$-free and Co^{2+}-substituted Ca^{2+}-free saline. A2, A3 and A4: 20 min after administration of paeonol 150 µmol/L, 500 µmol/L and 1.5 mmol/L, respectively. B1: Steady-state outward currents of the RP4 neuron in TEA (50 mmol/L)-containing Na$^+$-free and Co^{2+}-substituted Ca^{2+}-free saline. B2: 20 min after administration of paeonol (500 µmol/L), from B1. C1 and C2: Effects of paeonol (500 µmol/L and 1.5 mmol/L) on the current-voltage relationships of the steady-state outward currents measured at 500 ms of the RP4 neuron in Co^{2+}-substituted Ca^{2+}-free and Na$^+$-free solution, respectively. The closed circle (●) in C1 and C2 represents the *I–V* relationship before paeonol (500 µmol/L) application. The open circle (○) in C1 and closed square (■) in C2 represent the *I–V* relationship at 20 min after paeonol (500 µmol/L and 1.5 mmol/L) application, respectively ([b]*P*<0.05 *vs* control, *n*=5). C3: Effects of paeonol (500 µmol/L) on the current-voltage relationships of the steady-state outward currents measured at 500 ms of the RP4 neuron in TEA (50 mmol/L)-containing Co^{2+}-substituted Ca^{2+}-free and Na$^+$-free solution. The closed circle (●) and open circle (○) in C3 represent the *I–V* relationship before and at 20 min after paeonol (500 µmol/L) application, respectively ([b]*P*<0.05 *vs* the data in TEA 50 mmol/L). However, when neurons were pretreated with TEA, paeonol was less effective at decreasing the outward current.

significantly decreased the current under test potentials ranging from -50 mV to 50 mV; the current amplitude measured at 500 ms was reduced by 84.4±0.7 nA (by 32.3%±2.6% *vs* controls, paired *t*-test, *P*<0.05, *n*=5) at a test potential of 50 mV.

The effects of paeonol (500 µmol/L) on the steady-state outward current in Na$^+$-free and Co^{2+}-substituted Ca^{2+}-free solution containing TEA (50 mmol/L) are shown in Figure 6B. The corresponding amplitudes of the steady-state outward current were measured after 500 ms of the outward current, and the steady-state current-voltage (*I–V*) relationships are shown in Figure 6C3. After a 20-min application of paeonol (500 µmol/L), the currents at test potentials between -50 and

30 mV were not significantly affected, but the currents at test potentials of 40 and 50 mV were slightly decreased. The current amplitude at 500 ms was reduced by 10.7±0.5 nA (by 6.6%±0.4% *vs* controls, paired *t*-test, *P*<0.05, *n*=4) at a test potential of 50 mV.

Paeonol (500 µmol/L) decreased the steady-state current in a Na$^+$-free and Co^{2+}-substituted Ca^{2+}-free solution, but only slightly decreased the steady-state current in RP4 neurons pretreated with TEA (50 mmol/L) (Figure 6). These data suggest that the paeonol-elicited decrease in the steady-state current in the Na$^+$-free and Co^{2+}-substituted Ca^{2+}-free solution is mainly due to a decrease in the TEA-sensitive current.

Effects of 4-AP, TEA and charybdotoxin on the spontaneous action potentials of the RP4 neuron

To elucidate the role of I_{KD}, I_A, and $I_{K(Ca)}$ in the generation of the action potential bursts elicited by paeonol, we tested the effects of 4-AP, TEA and charybdotoxin on spontaneous action potentials. An example of the effects of 4-AP (5 mmol/L) is shown in Figure 7B. Application of 4-AP, an inhibitor of I_A[24], failed to elicit action potential bursts. Table 1 details the RMPs, frequencies and amplitudes of spontaneously generated action potentials after a 40-min treatment with 4-AP (5 mmol/L).

TEA (50 mmol/L) elicited bursts of action potentials in the RP4 neurons, as shown in Table 1. An example of the effects of TEA on the spontaneous firing of action potentials is shown in Figure 7D.
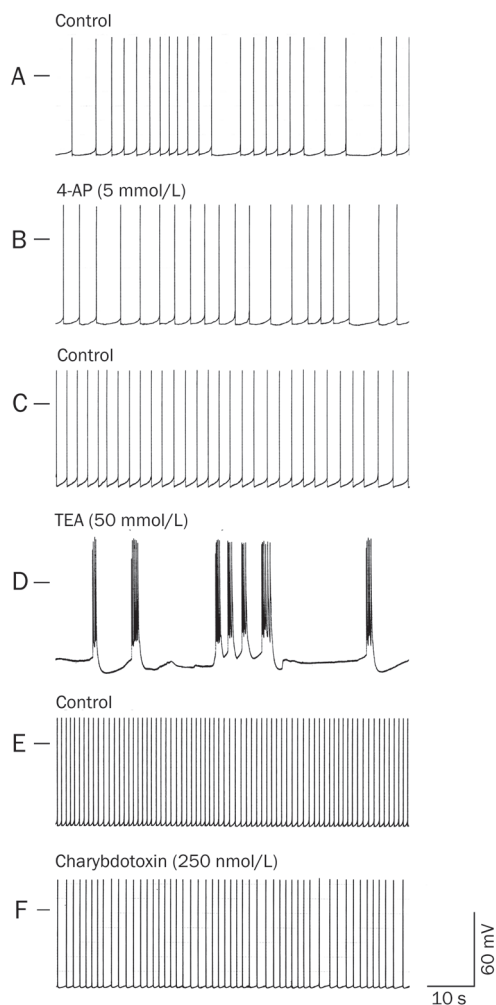
Charybdotoxin is a $I_{K(Ca)}$ inhibitor and reportedly inhibits $I_{K(Ca)}$ in the *Aplysia* neuron at concentrations between 100 nmol/L and 300 nmol/L[25]. We therefore treated RP4 neurons with 250 nmol/L of charybdotoxin. After 40 min, the RMPs, the frequencies and amplitudes of spontaneously generated action potentials remained unchanged from baseline. No bursting activity of potentials was observed (Figure 7F).

Effects of TEA on paeonol-elicited action potential bursts

To test whether the I_{KD} is involved in the generation of action potential bursts elicited by paeonol (500 µmol/L), we assessed the effects of TEA (5 mmol/L), an I_{KD} blocker. As shown in Table 1 and Figure 8, TEA (5 mmol/L) reduced the frequency of action potentials but failed to elicit action potential bursts.
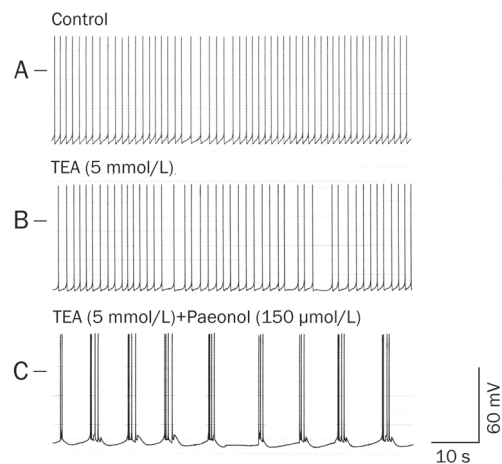

Figure 7. Effects of 4-AP (5 mmol/L), TEA (50 mmol/L) and charybdotoxin (250 nmol/L) on spontaneous action potentials of the RP4 neuron. A and B, C, and D, E, and F were recorded from 3 different RP4 neurons. A, C, and E: Control; the spontaneous action potentials of the RP4 neuron. B, D, and F: The potentials at 40 min after perfusion with 4-AP (5 mmol/L), TEA (50 mmol/L) and charybdotoxin (250 nmol/L), respectively. The upper left-hand horizontal bar indicates the membrane potential at 0 mV. Note that while action potential bursts were not elicited by 4-AP (5 mmol/L) or charybdotoxin (250 nmol/L), they were elicited by TEA (50 mmol/L).

Figure 8. Effects of co-application of TEA (5 mmol/L) plus paeonol (150 µmol/L) in the RP4 neuron. (A) Control; spontaneous action potentials of the RP4 neuron. (B) Potentials at 30 min after administration of TEA (5 mmol/L). (C) Potentials at 20 min after further incubation with paeonol (150 µmol/L) and TEA (5 mmol/L). The left-hand horizontal bar indicates the membrane potential at 0 mV. Note that co-application of TEA (5 mmol/L) plus paeonol (150 µmol/L) elicited action potential bursts.

The facilitative effects of TEA were tested in RP4 neurons pretreated with a low concentration of paeonol (150 µmol/L) (Figure 8). Whereas paeonol at 150 µmol/L alone failed to elicit action potential bursts, the coadministration of paeonol (150 µmol/L) with TEA (5 mmol/L) elicited action potential bursts after 20 min.

Discussion

This study investigated the effects of paeonol on the electrophysiological behavior of the central RP4 neuron in the giant African snail (*Achatina fulica* Ferussac). The neuron exhibited spontaneous regular firing of action potentials. No bursts of action potential activity were found in control RP4 neurons, whereas extracellular application of paeonol (500 µmol/L and 1.5 mmol/L) reversibly elicited bursts of action potential spikes in a dose-dependent manner (Figure 1). The highest

concentration of paeonol (1.5 mmol/L) elicited more remarkable bursting behavior patterns compared with those observed after 500 µmol/L. The bursting pattern elicited by paeonol (500 µmol/L) was not abolished after continuous perfusion with Co^{2+}-substituted Ca^{2+}-free solution (Figure 2B2), although the amplitudes of the action potentials were reduced. These findings suggest that paeonol-elicited bursts of potential firing are not directly associated with calcium fluxes of the neuron.

It has been suggested that both PKA and PKC play a key role in the plasticity of the nervous system in vertebrates and invertebrates. PKA and PKC modulate the function of ion channels[26-28]. In previous studies, we found that PKA and PKC activity were associated with the generation of action potential bursts in the central RP4 snail neuron[16, 17].

The PKA inhibitor KT-5720 is used at concentrations of approximately 10 µmol/L as a research tool in snail and *Aplysia* neurons[12, 29]. The bisindolylmaleimide Ro 31-8220 is a structurally-related staurosporine analog, which acts as an ATP-competitive inhibitor of PKC and has been used extensively for studying the role of PKC in cell signaling[22]. Recently, Ro 31-8220 (1–10 µmol/L)[22] has been used in snail[30] and *Aplysia* neurons[27]. In our previous work, pretreatment with KT-5720 (10 µmol/L) and Ro 31-8220 (20 µmol/L) prevented the induction of action potential bursts elicited by methamphetamine (METH) and 3,4-methylenedioxymethamphetamine (MDMA), respectively[16]. In the present study, the action potential bursts elicited by paeonol (500 µmol/L) were not affected by KT-5720 (10 µmol/L; Figure 3) or Ro 31-8220 (20 µmol/L; Figure 3). These results suggest that the action potential bursts elicited by paeonol are not associated with PKA and PKC activity.

Ionic currents play an important role in the firing of action potential bursts, as seen in the neurons of *Aplysia californica*[31], *Euhadra peliomphala*[32], and *Drosophila*[33]. To understand the mechanism underlying paeonol-elicited potential bursts, the effects of paeonol on ionic currents were investigated at a concentration of 500 µmol/L. As shown in Figure 4, paeonol had no remarkable effects on the total inward currents.

In the RP4 neuron, the I_A is sensitive to 4-AP, the I_{KD} is sensitive to TEA, and the $I_{K(Ca)}$ is sensitive to extracellular Co^{2+} ions[13]. These findings are consistent with other research involving snail or *Aplysia* neurons [19, 34, 35].

The measurement of the I_A followed Thompson's method[19]. The currents were elicited by 200 ms test potentials of -60 mV, -50, -40, and -30 mV from a holding potential of -100 mV. At 20 min after paeonol application (500 µmol/L and 1.5 mmol/L), the peak I_A current was decreased (Figure 5). The I_A current was completely abolished if the bath solution was infused with 4-AP (5 mmol/L) for 20 min. Notably, perfusion with 4-AP (5 mmol/L) elicited no bursts of action potentials in the RP4 neuron (Figure 7B), a finding that is consistent with our previous study[13]. These results suggest that the effects of paeonol on the I_A current may not be directly related to its effects on bursts of action potentials.

To measure the I_{KD} current, the steady-state outward current was elicited with 500-ms-long voltage clamp steps in a

Na+-free and Co^{2+}-substituted Ca^{2+}-free solution[36]. Under this condition, the $I_{K(Ca)}$, I_{Ca}, and I_{Na} were removed. To identify the I_{KD}, TEA was applied. The amplitude at 500 ms of outward current was significantly reduced after perfusion with TEA (50 mmol/L). The sensitivity of the outward current to TEA indicated that this is a type of I_{KD}. Notably, a sustained residual current was insensitive to TEA[19].

We also sought to determine whether paeonol affected the I_{KD}. Our results indicate that paeonol at 500 µmol/L decreased the steady-state current in Na+-free and Co^{2+}-substituted Ca^{2+}-free solution but only slightly decreased the steady-state current when the RP4 neurons were pretreated with TEA 50 mmol/L (Figure 6). This result suggests that paeonol decreases the TEA-sensitive I_{KD}. In our experiments, paeonol significantly decreased the outward current, including the I_A and the I_{KD}, but did not affect the total inward current. It may be that paeonol has greater potency on potassium currents in this preparation.

To further test whether inhibition of I_{KD} is involved in the generation of action potential bursts, we examined the effects of TEA on action potential bursting. As in our previous work, we found that TEA elicited action potential bursts at a concentration of 50 mmol/L (Figure 7D), but not at 5 mmol/L (Figure 8B)[13]. In the present study, paeonol (150 µmol/L) alone failed to elicit action potential bursts (Figure 1B), whereas its co-application with TEA (5 mmol/L) did elicit action potential bursts (Figure 8C). These data suggest that inhibition of I_{KD} facilitates the effects of paeonol; the TEA-sensitive I_{KD} helps to generate paeonol-elicited action potential bursts.

The $I_{K(Ca)}$ current plays an important role in the regulation of neuronal excitability[37]. To further clarify the role of the $I_{K(Ca)}$ current in the generation of action potential bursts, we tested the effects of charybdotoxin; this agent failed to elicit action potential bursts (Figure 7F). Notably, perfusion with Co^{2+}-substituted Ca^{2+}-free solution failed to elicit action potential bursts in the RP4 neuron (Figure 2). These findings suggest that the $I_{K(Ca)}$ current may not be involved in the generation of action potential bursts.

In animal models, paeonol showed anxiolytic-like effects at a dose of 17.5 mg/kg (*po*), and at doses of 50 and 100 mg/kg (*po*) in mice successfully reduced oxidative stress, cognitive impairment and neurotoxicity induced by *D*-galactose (50 mg·kg^{-1}·d^{-1})[9, 38]. At doses of 30 and 100 mg/kg (*ip*), paeonol inhibited carrageenan-evoked thermal hyperalgesia in rats[39]. In mice, maximum plasma levels were around 16.3 µmol/L after oral dosing with 20 mg/kg paeonol[40].

In vitro studies have used varying concentrations. At concentrations up to 500 µg/mL (around 3 mmol/L), paeonol has not shown any cytotoxicity in normal human endothelial cells as assessed by a trypan blue dye exclusion test. In the concentration range of 125 to 500 µg/mL (around 750 µmol/L to 3 mmol/L), paeonol significantly inhibited proliferation of basic fibroblast growth factor (bFGF)-stimulated human umbilical vein endothelial cells (HUVECs)[41]. At concentrations ranging from 24 µmol/L to 1.5 mmol/L, paeonol inhibited cell proliferation in HT-29 cells[42] and protected rat neurons from

oxygen-glucose deprivation-induced injury at 0.2–5 µmol/L[8]. In addition, paeonol (15.63–62.5 mg/L) in combination with cisplatin has shown a synergistic growth-inhibitory and apoptosis-inducing effect in two human hepatoma cell lines[43].

In investigations seeking clarification of how paeonol affects ionic function in excitable tissue, concentrations of 160 µmol/L and 640 µmol/L successfully decreased the action potential upstroke phase in guinea pig ventricular cells[7]. At concentrations ranging from 25 µg/mL to 400 µg/mL (150 µmol/L–2.4 mmol/L), paeonol inhibited the delayed outward K^+ current and, to a lesser extent, the I_{Na} in NG108-15 cells[44]. The data from our present study indicate that paeonol at a concentration of ≥500 µmol/L reversibly elicits action potential bursts of the central RP4 neuron. The concentration used in the present study may be higher than that of the plasma concentration *in vivo* and may be associated with toxic effects.

An identifiable unit of epileptiform activity in the mammalian central nervous system (CNS) is the interictal spike. The intracellular correlate of the interictal spike is an overt depolarization, termed the paroxysmal depolarizing shift (PDS), that results in the initiation of a high-frequency burst of action potentials followed by a period of hyperpolarization[45–47]. Research has revealed that convulsants, such as pentylenetetrazol (PTZ), induce bursts of action potentials in snail central neurons[32, 48, 49], a response that closely resembles the PTZ-induced PDS in the cerebral cortical neurons of mammals[50]. In our previous studies, CNS stimulants, such as *d*-amphetamine, cocaine and methamphetamine, elicited *in vitro* action potential bursts in the central RP4 neuron of the African snail, *Achatina fulica* Ferussac[13, 15, 16]. Interestingly, high doses of these substances induce convulsions in animals and in humans. The present study demonstrated that paeonol at 500 µmol/L induced bursts of action potential in neurons. Our data indicate that paeonol at high doses may have a pro-epileptic effect. Patients should be closely supervised if the Moutan cortex of *Paeonia suffruticosa* Andrews (MC) is combined with a CNS stimulant.

Conclusion

The present study aimed to elucidate the effects of paeonol on membrane potentials and ionic currents in the central RP4 neuron using the conventional two-electrode voltage clamp technique. Our results indicate that paeonol elicits a bursting firing pattern in action potentials that is closely related to the inhibitory effects on the I_{KD}.

Author contribution

Yi-hung CHEN: collection and assembly of data, manuscript writing; Pei-lin LIN: conception and design; Hui-yu HSU: collection and assembly of data; Ya-ting WU: revision of the manuscript; Dah-yuu LU: conception and design; Han-yin YANG: collection and assembly of data; Shiang-suo HUANG: collection and assembly of data; Ching-liang HSIEH: conception and design; Jaung-geng LIN: conception and design, manuscript writing.

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