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\textsuperscript{[9]}. These results suggest that paeonol possesses antiaging 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\textsuperscript{[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\textsuperscript{[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\textsuperscript{[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\textsuperscript{[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\textsuperscript{[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 digitizing unit (Digidata 1440, Axon Instruments) and analyzed using pCLAMP software.

Na\(^+\)-free solution was used to measure the fast-inactivating K\(^+\) current (I\(_{Na}\)). The currents were elicited by 200 ms test potentials of -60, -50, -40, and -30 mV from a holding potential of -100 mV\textsuperscript{[19]}. A P4/4 leak subtraction subpulse routine supplied by the pCLAMP software was used to measure the I\(_{Na}\).

For measuring the I\(_{KCa}\), 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\(_{KCa}\)). 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\textsuperscript{[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\textsuperscript{[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
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 Co²⁺-substituted Ca²⁺-free solution on paeonol-elicited potential changes of the RP4 neuron

To test the effects of Co²⁺-substituted Ca²⁺-free solution on paeonol-elicited changes in action potential bursts, the RP4 neuron underwent 30 min of treatment with Co²⁺-substituted Ca²⁺-free solution alone (Figure 2A2) or in combination with paeonol (Figure 2B2). Co²⁺-substituted Ca²⁺-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 Co²⁺-substituted Ca²⁺-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

Table 1. Effects of paeonol, Ro 31-8220, KT-5720, Co²⁺-substituted Ca²⁺-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, Co²⁺-substituted Ca²⁺-free solution and TEA on paeonol-elicited action potential bursts. Values are expressed as the mean±SEM. (n being the number of neurons tested). *P<0.05 vs paeonol (500 µmol/L)-elicited action potential bursts. †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±1.5                       | 8.7±1.7*                       |
| Paeonol (1.5 mmol/L)            | 3  | -59.3±2.3| 83.3±9.6                           |                                       |                               |                                 |
| 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  | 3  | -54.0±2.3| 100.3±4.5                          |                                       |                               |                                 |
| 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     | 3  | -61.7±3.5| 96.1±7.3                           | 7.0±1.5                               | 4.3±0.3                       |                                 |
| Paeonol in Ca²⁺-free Control    | 3  | -49.8±3.5| 65.7±3.5                           | 22.7±5.4                              |                               |                                 |
| Ca²⁺-free                      | 3  | -58.3±1.5| 73.5±5.3                           | 2.7±1.2                               | 17.3±5.3*                    |                                 |
| Paeonol (500 µmol/L) in Ca²⁺-free| 3  | -59.3±3.3| 82.3±5.2                           | 51.3±5.3                              |                               |                                 |
| Paeonol+TEA Control             | 3  | -57.5±1.4| 86.0±3.2                           | 29.5±1.3*                             |                               |                                 |
| TEA (5 mmol/L)                 | 4  | -56.3±0.7| 92.7±5.6                           | 14.7±6.7                              | 3.7±0.9                       |                                 |
| Paeonol (500 µmol/L)+TEA        | 4  | -56.3±1.6| 91.5±6.1                           | 49.0±14.8                             |                               |                                 |
| KT-5720 (10 µmol/L) + Paeonol   | 4  | -58.0±4.0| 84.4±5.6                           | 10.5±5.5                              | 3.1±0.7                       |                                 |
| 4-AP Control                    | 3  | -59.0±1.7| 87.3±2.6                           | 49.6±6.0                              |                               |                                 |
| 4-AP (5 mmol/L)                | 4  | -57.6±1.2| 88.7±3.2                           | 39.3±1.5                              |                               |                                 |
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)\(^2\), 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)\(^2\). 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.
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, -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 Ca²⁺ ions decreased the amplitude of the total inward currents[13, 23]. Both the $I_{Ca}$ and $I_{Na}$ 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%±3.1%, 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 Co²⁺-substituted Ca²⁺-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 Co²⁺-substituted Ca²⁺-free solution are shown in
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...
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 Co²⁺-substituted Ca²⁺-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 Co²⁺-substituted Ca²⁺-free solution, but only slightly decreased the steady-state current in RP4 neurons pre-treated 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 Co²⁺-substituted Ca²⁺-free solution is mainly due to a decrease in the TEA-sensitive current.

Figure 6. Effects of paeonol on steady-state outward currents of the RP4 neuron in Na⁺-free and Co²⁺-substituted Ca²⁺-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 Co²⁺-substituted Ca²⁺-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 Co²⁺-substituted Ca²⁺-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 Co²⁺-substituted Ca²⁺-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 (*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 Co²⁺-substituted Ca²⁺-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 (*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.
Effects of 4-AP, TEA and charybdotoxin on the spontaneous action potentials of the RP4 neuron

To elucidate the role of $I_{K_D}$, $I_A$, and $I_{K(Ca)}$ in the generation of the action potential bursts elicited by paenonol, 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 paenonol-elicited action potential bursts

To test whether the $I_{K_D}$ is involved in the generation of action potential bursts elicited by paenonol (500 µmol/L), we assessed the effects of TEA (5 mmol/L), an $I_{K_D}$ 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.

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

Discussion

This study investigated the effects of paenonol 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 paenonol (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 Co2+-substituted Ca2+-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[12–15]. 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[21]. 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 (MET) and 3,4-methyleneoxymethamphetamine (MDMA), respectively[20]. 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_KCa is sensitive to extracellular Ca2+ 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 Co2+-substituted Ca2+-free solution[36]. Under this condition, the I_KCa, 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[39].

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 Co2+-substituted Ca2+-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_KCa current plays an important role in the regulation of neuronal excitability[17]. To further clarify the role of the I_KCa 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 Co2+-substituted Ca2+-free solution failed to elicit action potential bursts in the RP4 neuron (Figure 2). These findings suggest that the I_KCa 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[6]. 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[49].

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 \( I_{\text{Kd}} \) in NG108-15 cells[48]. 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 \( \text{in vitro} \) 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 \( \text{d}-\)amphetamine, cocaine and methamphetamine, elicited \( \text{in vitro} \) action potential bursts in the central RP4 neuron of the African snail, \( \text{Achatina fulica} \) 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 \( \text{Paeonia suffruticosa} \) 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 \( I_{\text{Kd}} \).

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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-ying LU: conception and design; Han-yin YANG: conception and design; and, to a lesser extent, the \( I_{\text{Kd}} \) in NG108-15 cells[48]. 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 \( \text{in vitro} \) and may be associated with toxic effects.

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References

1. Hirai A, Terano T, Hamazaki T, Sajiki J, Saito H, Tahara K, et al. Studies on the mechanism of antiaggregatory effect of Moutan Cortex. Thromb Res 1983; 31: 29–40.
2. Lin HC, Ding HY, Ko FN, Teng CM, Wu YC. Aggregation inhibitory activity of minor acetophenones from Paeonia species. Planta Med 1999; 65: 595–9.
3. Harada M, Yamashita A, Abara M. Pharmacological studies on the root bark of \( \text{Paeonia moutan} \). II. Anti-inflammator effect, preventive effect on stress-induced gastric erosion, inhibitory effect on gastric juice secretion and other effects of paeonol. Yakugaku Zasshi 1972; 92: 750–6.
4. Zhang HY, Ge N, Zhang ZY. Theoretical elucidation of activity differences of five phenolic antioxidants. Acta Pharmacol Sin 1999; 20: 363–6.
5. Zhang WG, Zhang ZS. Anti-ischemia reperfusion damage and anti-lipid peroxidation effects of paeonol in rat heart. Yao Xue Xue Bao 1994; 29: 145–8.
6. Zhang GQ, Hao XM, Zhou PA, Wu CH. Effect of paeonol on L-type calcium channel in rat ventricular myocytes. Methods Find Exp Clin Pharmacol 2003; 25: 281–5.
7. Ma YL, Bates S, Gumey AM. The effects of paeonol on the electrophysiological properties of cardiac ventricular myocytes. Eur J Pharmacol 2006; 545: 87–92.
8. Wu JB, Song NN, Wei XB, Guan HS, Zhang XM. Protective effects of paeonol on cultured rat hippocampal neurons against oxygen-glucose deprivation-induced injury. J Neurol Sci 2008; 264: 50–5.
9. Zhong SZ, Ge QH, Qu R, Li Q, Ma SP. Paeonol attenuates neurotoxicity and ameliorates cognitive impairment induced by \( \text{d}-\)galactose in ICR mice. J Neurol Sci 2009; 277: 58–64.
10. Kerkut GA, Lambert JD, Gayton RJ, Loker JE, Walder RJ. Mapping of nerve cells in the suboesophageal ganglia of \( \text{Helix aspersa} \). Comp Biochem Physiol A Comp Physiol 1975; 50: 1–25.
11. Takeuchi H, Araki Y, Emaduddin M, Zhang W, Han XY, Salunga TL, et al. Identifiable Achatina giant neurones: their localizations in ganglia, axonal pathways and pharmacological features. Gen Pharmacol 1999; 32: 7–32.
12. Lin CH, Tsai MC. The modulation effects of \( \text{d}-\)amphetamine and procaine on the spontaneously generated action potentials in the central neuron of snail, Achatina fulica Ferussac. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 1983; 78: 551–560.
13. Chen YH, Lin CH, Lin PL, Tsai MC. Cocaine elicits action potential bursts in a central snail neuron: the role of delayed rectifying K+ current. Neuroscience 2006; 138: 257–80.
14. Onizuka S, Kasaba T, Takasaki M. The effect of lidocaine on cholinergic neurotransmission in an identified reconstructed synapse. Anesth Analg 2008; 107: 1236–42.
15. Tsai MC, Chen YH. Bursting firing of action potentials in central snail neurons elicited by \( \text{d}-\)amphetamine: role of the electrogenic sodium pump. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 1995; 111: 131–41.
16. Lin PL, Tsai MC, Lu GL, Lu DY, Chuang CM, Yang HY, et al. Ecstasy and methamphetamine elicit action potential bursts via different
mechanisms in a central snail neuron. Neurotoxicology 2010; 31: 26–41.

17 Tsai MC, Chen YH. (+/-)3,4-Methylenedioxyamphetamine elicits action potential bursts in a central snail neuron. Exp Neurol 2007; 203: 423–44.

18 Arvanov VL, Liou HH, Chang YC, Chen RC, Peng FC, Ling KH, et al. Interactions of anticholinesterases with Achatina fulica acetylcholine responses and electrogenic sodium pump. Neuroscience 1994; 62: 581–6.

19 Thompson SH. Three pharmacologically distinct potassium channels in molluscan neurones. J Physiol 1977; 265: 465–88.

20 Sun XP, Funase K, Takeuchi H. Ionic current of an identifiable giant neurone, d-RPLN, of an African giant snail (Achatina fulica Ferussac), measured under voltage clamping — II. Outward currents. Comp Biochem Physiol A Comp Physiol 1987; 88: 707–14.

21 Kim MS, Cheong YP, So HS, Lee KM, Son Y, Lee CS, et al. Regulation of cyclic AMP-dependent response element-binding protein (CREB) by the nociceptin/orphanin FQ in human dopaminergic SH-SY5Y cells. Biochem Biophys Res Commun 2002; 291: 663–8.

22 Beltman J, McCormick F, Cook SJ. The selective protein kinase C inhibitor, Ro-31-8220, inhibits mitogen-activated protein kinase phosphatase-1 (MKP-1) expression, induces c-Jun expression, and activates Jun N-terminal kinase. J Biol Chem 1996; 271: 27018–24.

23 Chen YH, Tsai MC. Action potential bursts in central snail neurones elicited by d-amphetamine: roles of ionic currents. Neuroscience 2000; 96: 237–48.

24 Bukanova JV, Solntseva EI, Skrebbitsky VG. Selective suppression of the slow-inactivating potassium currents by nootropics in molluscan neurones. Int J Neurpsychopharmacol 2002; 5: 229–37.

25 Hermann A, Erxleben C. Charybdotoxin selectively blocks small Ca¬currents in Aplysia neurons. J Gen Physiol 1987; 90: 27–47.

26 Conn PJ, Strong JA, Azhdarian EM, Nairn AC, Greengard P, Kaczmarek LK. Protein kinase inhibitors selectively block phorbol ester- or forskolin-induced changes in excitability of Aplysia neurons. J Neurosci 1989; 9: 473–9.

27 Manseau F, Sossin WS, Castellucci VF. Long-term changes in excitability induced by protein kinase C activation in Aplysia sensory neurons. J Neurophysiol 1998; 79: 1210–8.

28 Su TR, Chen CH, Huang SJ, Lee CY, Su MC, Chen GH, et al. Functional study of the effect of phosphonates inhibitors on KCNQ4 channels expressed in Xenopus oocytes. Acta Pharmacol Sin 2009; 30: 1220–6.

29 Nakayama T, Harada N, Asano M, Nomura N, Saito T, Mishima A, et al. Atrial natriuretic peptide reduces ischemia/reperfusion-induced spinal cord injury in rats by enhancing sensory neuron activation. J Pharmacol Exp Ther 2007; 322: 582–90.

30 Nikitin VP, Kozrey SA. Protein kinase C is selectively involved in the mechanisms of long-term synaptic plasticity. Bull Exp Biol Med 2005; 139: 639–42.

31 Smith TG Jr, Barker JL, Gainer H. Requirements for bursting pacemaker potential activity in molluscan neurones. Nature 1975; 253: 450–2.

32 Onozuka M, Kubo KY, Ozono S. The molecular mechanism underlying pentylenetetrazole-induced bursting activity in Euhadra neurons: involvement of protein phosphorylation. Comp Biochem Physiol C 1991; 100: 423–32.

33 Zhao ML, Wu CF. Alterations in frequency coding and activity dependence of excitability in cultured neurons of Drosophila memory mutants. J Neurosci 1997; 17: 2187–99.

34 Staras K, Gyorl J, Kemenes G. Voltage-gated ionic currents in an identified modulatory cell type controlling molluscan feeding. Eur J Neurosci 2002; 15: 109–19.

35 Sakakibara M, Okuda F, Nomura K, Watanabe K, Meng H, Horikoshi T, et al. Potassium currents in isolated statocyst neurons and RPeD1 in the pond snail, Lymnaea stagnalis. J Neurophysiol 2005; 94: 3884–92.

36 Kraiz D, Singh S. Selective blockade of the delayed rectifier potassium current by tacrine in Drosophila. J Neurobiol 1997; 32: 1–10.

37 Latorre R, Oberhauser A, Labarca P, Alvarez O. Varieties of calcium-activated potassium channels. Annu Rev Physiol 1989; 51: 385–99.

38 Mi XJ, Chen SW, Wang WJ, Wang R, Zhang YJ, Li WJ, et al. Anxiolytic-like effect of paeonol in mice. Pharmacol Biochem Behav 2005; 81: 683–7.

39 Chou TC. Anti-inflammatory and analgesic effects of paeonol in carrageenan-evoked thermal hyperalgesia. Br J Pharmacol 2003; 139: 1146–52.

40 Wu X, Chen H, Chen X, Hu Z. Determination of paeonol in rat plasma by high-performance liquid chromatography and its application to pharmacokinetic studies following oral administration of Moutan cortex decoction. Biomed Chromatogr 2003; 17: 504–8.

41 Kim SA, Lee HJ, Ahn KS, Lee EO, Choi SH, Jung SJ, et al. Paeonol exerts anti-angiogenic and anti-metastatic activities through down-modulation of Akt activation and inactivation of matrix metallo-proteinases. Biol Pharm Bull 2009; 32: 1142–7.

42 Ye JM, Deng T, Zhang JB. Influence of paeonol on expression of COX-2 and p27 in HT-29 cells. World J Gastroenterol 2009; 15: 4410–4.

43 Xu SP, Sun GP, Shen YX, Peng WR, Wang H, Wei W. Synergetic effect of combining paeonol and cisplatin on apoptotic induction of human hepatoma cell lines. Acta Pharmacol Sin 2007; 28: 869–78.

44 Hu Q, Shi YL. Inhibition of voltage-gated K+, Na+, and Ca2+ currents in neuroblasts x glia hybrid cells by paeonol. Sheng Li Xue Bao 1994; 46: 575–80.

45 McCormick DA, Contreras D. On the cellular and network bases of epileptic seizures. Annu Rev Physiol 2001; 63: 815–46.

46 Jefferys JG. Nonsynaptic modulation of neuronal activity in the brain: electric currents and extracellular ions. Physiol Rev 1995; 75: 689–723.

47 Jefferys JG. Experimental neurobiology of epilepsies. Curr Opin Neurol 1994; 7: 113–22.

48 Onozuka M, Imai S, Sugaya E. Pentylenetetrazole-induced bursting activity and cellular protein phosphorylation in snail neurones. Brain Res 1986; 362: 33–9.

49 Sugaya E, Furuichi H, Takagi T, Kajiwara K, Komatsubara J. Intracellular calcium concentration during pentylenetetrazol-induced bursting activity in snail neurons. Brain Res 1987; 416: 183–6.

50 Szymusiak R, Shouse MN, McGinty D. Brainstem stimulation during sleep evokes abnormal rhythmic activity in thalamic neurons in feline penicillin epilepsy. Brain Res 1996; 713: 253–60.