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

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Abstract

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 effect of paeonol on the RP4 neuron.

Results : The RP4 neuron generated spontaneous action potentials. Bath application of paeonol at a concentration of 500 μM or higher reversibly elicited action potential bursts of the central RP4 neuron in a concentration-dependent manner. The action potential bursts were not blocked after immersing the neurons in Co^{2+}-substituted Ca^{2+}-free solution. The action potential bursts were not affected by pretreatment with the protein kinase A (PKA) inhibitor, KT-5720, nor by the protein kinase C (PKC) inhibitor, Ro 31-8220. Voltage-clamp studies revealed that while paeonol at 500 μM had no remarkable effects on the total inward currents~~, this concentration decreased the steady-state outward currents~~. Further analysis on the outward currents revealed that paeonol decreased the delayed rectifying current (I_{KD}) and the fast-inactivating K^+ current (I_A). Application of 4-aminopyridine (4-AP), an inhibitor of I_A, and charybdotoxin, an inhibitor of the Ca^{2+}-activated K^+ current ($I_{K(Ca)}$), failed to elicit action potential bursts, whereas tetraethylammonium chloride (TEA), a blocker of the

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I_{KD}, successfully elicited action potential bursts. TEA at a lower concentration of 5 mM facilitated the induction of action potential bursts elicited by paeonol.

Conclusion: Paeonol elicits a bursting firing pattern of action potentials in the RP4 neuron. This activity is closely related to the inhibitory effects of paeonol on the I_{KD}.

Keywords: paeonol, neuron, snail, action potential bursts, ionic currents

Introduction

The Chinese have used herbs for a wide variety of medical treatments for several thousand years. Moutan cortex, the root bark of *Paeonia suffruticosa* Andrews is a commonly used Chinese herb and has long been used for its antipyretic and anti-inflammatory effects in Traditional Chinese Medicine [1-3]. 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, besides the ability to suppress ADP or collagen-induced human blood platelet aggregation [4-7].

Recent pharmacological experiments have shown that paeonol protects against reperfusion-induced myocardium damage [8]. Paeonol has also been reported to block the L-type calcium current in cardiac myocytes, thereby decreasing the excitability of cardiac tissue [9]. 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 [10].

In other research, paeonol has indicated neuroprotective effects. For example, paeonol has been shown to protect rat neurons from oxygen–glucose deprivation-induced injury, alleviating the morphological damage and increasing neuron viability [11]. Paeonol has ameliorated neuronal damage in both the

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hippocampus and temporal cortex in d-galactose-treated mice [12]. 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 the relative simplicity of the molluscan neuronal network has proven it to be the ideal *in vitro* preparation for electrophysiological and neuropharmacological studies [13-15]. The neurons in ganglia can be identified for investigations into drug-related effects on the same neuron [16]. Snail ganglia contain many identifiable neurotransmitters and receptors, and their neurons are used for biological studies [14, 17-18]. In our previous studies, CNS stimulants such as d-amphetamine, cocaine and methamphetamine were found to elicit *in vitro* action potential bursts in the central Right Parietal 4 (RP4) neuron of the African snail, *Achatina fulica* Ferussac [17, 19-20].

The effects of paeonol on neuronal excitability have not been extensively studied. The present study aimed to examine the effects of paeonol on membrane potentials and ionic currents in the central RP4 neuron, using the conventional two-electrode

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voltage clamp technique. Our results indicate that paeonol elicits a bursting firing pattern in action potentials. The generation of action potential bursts elicited by paeonol is closely related to the inhibitory effects on the delayed rectifying current (I_{KD}).

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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 = 2ml) and removed from the connective tissue sheath to allow easy identification and penetration by microelectrodes [17, 20-21].

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 3M potassium chloride (KCl). The experimental chamber was perfused with control solution, i.e. (in mM) NaCl (85), KCl (4), $CaCl_2$ (8), $MgCl_2$ (7), Tris-HCl (10) at a pH of 7.6 and 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) was greater than -50 mV, the time constant at around 5-8 msec and the rate of rise of the action potentials at around 5-8 V/sec [22].

The ionic currents of the central snail neurons were measured by the two-electrode voltage clamp method. Two microelectrodes were penetrated 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 3M KCl. All potentials

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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 [23]. The measurement of I_A used a P/4 leak subtraction subpulse routine, as supplied by pCLAMP software.

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 ($I_{K(Ca)}$). The currents of the RP4 neuron 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 3M 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 mM) of Tris were added to replace Na^+ ions, (c) Co^{2+}-substituted Ca^{2+}-free solution: equimolar amounts (8 mM) of Co^{2+} were added to replace Ca^{2+} ions, (d) Na^+-free and Co^{2+}-substituted Ca^{2+}-free solution: equimolar amounts of Tris (85 mM) and Co^{2+} (8 mM) were added to replace Na^+ and Ca^{2+} ions, respectively. Tetraethylammonium chloride (TEA)-containing solution was prepared by replacing equimolar quantities of NaCl or Tris-Cl with TEA [24].

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Paeonol (2'-Hydroxy-4'-methoxyacetophenone), 4-aminopyridine (4-AP), tetraethylammonium chloride (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), while paeonol was prepared in ethanol. The presence of DMSO (≤0.1%) or ethanol (≤1%) did not affect the RMPs, amplitude or frequency of the spontaneous firing of action potentials in the RP4 neuron.

The statistical significances of the differences between the amplitude and frequency of the action potentials, RMPs and currents after various treatments and the pre-drug controls were determined by the Student's paired *t*-test. Differences were considered significant at $p<0.05$.

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Results

~~*The electrical characteristics of the identifiable RP4 neuron*~~

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 [19].

The effects of paeonol (150 μM, 500 μM and 1.5 mM) on the spontaneous firing action potentials of the central neuron (RP4) are shown in Figure 1 and Table 1. paeonol at a concentration of 150 μM did not alter the action potential firing pattern of the RP4 neuron. As shown in Table 1, at 20 minutes after extracellular perfusion of paeonol (150 μM), the frequency of spontaneously firing action potentials and the RMP remained unchanged. No action potential bursts were observed, even after 1 hour of incubation. However, at concentrations of 500 μM and 1.5 mM, paeonol changed the action potential firing pattern of the RP4 neuron. Twenty minutes after extracellular perfusion of paeonol (500 μM), 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), lasting for 5-15 seconds each. The RMP, action potential amplitude and bursting

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frequencyand number of action potentials for each burst are shown in Table 1. The effect of paeonol continued throughout its application (for up to 3 hours).

At the highest concentrations of 1.5 mM, 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 increased compared with those of the bursts elicited by paeonol at 500 μM (Table 1 and Figure 1). After 30 minutes 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 the Co^{2+}-substituted Ca^{2+}-free solution on paeonol-elicited changes in the action potential bursts, the RP4 neuron underwent 30 minutes of treatment with Co^{2+}-substituted Ca^{2+}-free solution alone (Figure 2A2) or in combination with paeonol (Figure 2B2). At 30 minutes after perfusion with Co^{2+}-substituted Ca^{2+}-free solution, the RMP and the amplitude of action potentials were decreased (Table 1). No action potential bursts were elicited by the Co^{2+}-substituted Ca^{2+}-free solution (Figure 2A2).

The RP4 neuron was then perfused with Co^{2+}-substituted Ca^{2+}-free solution

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containing paeonol (500 μM) from normal physiological solution. After 30 minutes, action potential bursts were elicited (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 bursting elicited by paeonol at 500 μM in normal physiological solution.

It appears that removing extracellular calcium ions does not abolish the paeonol (500 μM)-induced bursting pattern of action potentials, although it does reduce the amplitude of action potentials.

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

In order to determine whether the cAMP-protein kinase A (PKA) signaling pathway is involved in the generation of action potential bursts elicited by paeonol, we tested the effects of the PKA inhibitor, KT-5720 [25-26]. As shown in Table 1 and Figure 3B, after 40 minutes of incubation with KT-5720 (10 μM), the frequency of the spontaneous action potentials, the RMP and amplitude of the action potentials did not differ from those of the control RP4 neuron.

As mentioned earlier, paeonol (500 μM) elicited action potential bursts in the RP4 neuron. The results of treatment with paeonol (500 μM) on RP4 neurons

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pretreated with KT-5720 are shown in Table 1 and Figure C. If KT-5720 (10 μM)-pretreated RP4 neurons were further incubated with paeonol (500 μM) for 20 minutes, action potential bursts were elicited.

It appears that pretreatment with KT-5720 fails to prevent paeonol-elicited action potential bursts.

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

In order 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 μM) [27-28]. After 40 minutes of incubation with Ro 31-8220 (20 μM), 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). When the Ro 31-8220-pretreated RP4 neurons were further incubated with paeonol (500 μM) for 20 minutes, action potential bursts occurred (Table 1 and Figure 3H).

It appears that pretreatment with Ro 31-8220 failed to abolish the generation of action potential bursts elicited by paeonol.

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

The fast ionic currents of the RP4 neurons clamped at 70 msec 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-msec-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 and the maximum peak inward current was observed after voltage stepping to the test potentials between -20 mV and -10 mV. As shown in our previous study, removing either extracellular Na^+ ion or Ca^{2+} ion concentrations decreased the amplitude of total inward currents [17, 29]. It appears that both the I_{Ca} and the I_{Na} contribute to the total inward currents of the RP4 neuron. As shown in Figure 4B, paeonol (500 μM) did not significantly decrease the fast inward currents in a series of voltage steps.

~~*Effects of paeonol on the steady-state currents of the RP4 neuron*~~

~~Five-second-long stepping pulses were used to measure the steady-state currents of the RP4 neuron. Currents were obtained by stepping from a holding potential of -60 mV to test potentials of -50 to 10 mV, at intervals of 10 mV in normal physiological solution. Example traces are shown in Figure 6A. The steady-state~~

~~currents were measured at 5 seconds after voltage stepping and the steady state current voltage (I-V) relationships are shown in Figure 6B. Steady state outward currents were observed if the positivity of the holding potential exceeded -20 mV. The outward current was increased if the command voltage was stepped to a higher positive potential.~~

~~The effects of paeonol (500 μM) on steady state currents are also shown in Figure 6. Compared with the pre-drug control values, the steady state currents were significantly decreased at 20 minutes after paeonol treatment if the positive values of the test potentials exceeded -20 mV.~~

Effects of paeonol on the I_A current

The measurement of I_A followed Thompson's method [23]. The currents were elicited by 200 msec 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 minutes (Figure 5F).

As shown in Figure 5, when neurons were perfused for 20 min with paeonol at 150 μM, the peak amplitude of I_A was not affected. However, the peak amplitude of I_A was decreased after 20 minutes' perfusion with paeonol at 500 μM and 1.5 mM (by

40.0 ± 6.7% ; n=4 and 85.7± 0.6%; n=3; respectively; at a test potential of -30 mV). It appears that paeonol at 150 μM has no effect upon the I_A, whereas paeonol at concentrations of 500 μM and 1.5 mM decrease 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-msec-long voltage clamp steps in Na^+-free and Co^{2+}-substituted Ca^{2+}-free solution (Figure 6A). In this condition, I_A and delayed rectifying K^+ (I_{KD}) components in the outward currents were identified by 4-AP and TEA, as in our previous study [17]. The amplitude of the outward current at 500 msec was significantly reduced (by 40.7 ± 3.1%; n=3; at a test potential of 50 mV) after perfusion with TEA (50 mM) for 40 minutes (Figure 6B). If 4-AP (5 mM) was applied to the bath solution for 20 minutes, 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 [17].

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 6A. The corresponding amplitude of the steady-state outward current as measured at 500 msec of the outward current, and the steady-state current-voltage (I–V) relationships, are shown in Figures

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6C1 and 6C2. When neurons underwent 20 minutes of treatment with paeonol at 150 μM, the steady-state outward current was not affected, whereas 20 minutes' treatment with paeonol at 500 μM 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 msec was reduced by 44.8 ± 5.2 nA (by 18.2 ± 1.8% from control; paired t-test, $p<0.05$, n=5) at a test potential of 50 mV. Further, 20 minutes' treatment with paeonol at 1.5 mM significantly decreased the current under test potentials in the range of -50 mV to 50 mV; the current amplitude measured at 500 msec was reduced by 84.4 ± 0.7 nA (by 32.3 ± 2.6 % from control, paired t-test, $p<0.05$, n=5) at a test potential of 50 mV.

The effects of paeonol (500 μM) on the steady-state outward current in Na^+-free and Co^{2+}-substituted Ca^{2+}-free solution containing TEA (50 mM) are shown in Figure 6B. The corresponding amplitude of the steady-state outward current was measured at 500 msec of the outward current and the steady-state current-voltage (I–V) relationships are shown in Figure 6C3. After 20 minutes of paeonol treatment at 500 μM, currents at test potentials of between -50 and 30 mV were not significantly affected, although currents at test potentials of 40 and 50 mV were slightly decreased. The current amplitude measured at 500 ms was reduced by 10.7 ± 0.5 nA (by 6.6 ± 0.4% from control, paired t-test, $p<0.05$, n=4) at a test potential of 50 mV.

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It appears that 500 μM paeonol 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 RP4 neurons were pretreated with TEA (50 mM) (Figure 6). These data suggest the decrease in the steady-state current in Na^+-free and Co^{2+}-substituted Ca^{2+}-free solution elicited by paeonol 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 mM) is shown in Figure 7A. Application of 4-AP, an inhibitor of I_A [30], failed to elicit action potential bursts. Table 1 lists the values for the RMP, the frequencies and amplitude of spontaneously generated action potentials in the presence of 4-AP (5 mM) after 40 minutes.

TEA (50 mM) 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

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potentials is shown in Fig. 7D.

Charybdotoxin is a $I_{K(Ca)}$ inhibitor and reportedly inhibits $I_{K(Ca)}$ in the *Aplysia* neuron, at concentrations of between 100 nM and 300 nM [31]. We therefore treated RP4 neurons with 250 nM charybdotoxin. After 40 minutes, the RMP, the frequencies and amplitude 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 the action potential bursts elicited by paeonol (500 μM), we tested the effects of TEA (5 mM), an I_{KD} blocker. As shown in Table 1 and Figure 8, TEA (5 mM) reduced the frequency of action potentials but failed to elicit action potential bursts.

The facilitatory effects of TEA were tested in RP4 neurons pretreated with a low concentration of paeonol (150 μM) (Figure 8). Whereas paeonol at 150 μM alone failed to elicit action potential bursts, when coadministered with TEA (5 mM), action potential bursts were observed after 20 minutes.

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Discussion

This study investigated the effects of paeonol on the electrophysiological behavior of the giant African snail (*Achatina fulica* Ferussac) central RP4 neuron. 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 μM and 1.5 mM) reversibly elicited bursts of action potential spikes in the RP4 neuron, in a dose-dependent manner (Figure 1). The higher concentration of paeonol (1.5 mM) elicited more remarkable bursting behavior patterns compared with those observed after 500 μM. The pattern of the bursts of potential elicited by paeonol (500 μM) 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 the 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 are able to modulate the function of ion channels. [32-34]. In our previous study, we found that PKA and PKC activity are associated with the generation of action potential bursts in the central RP4 snail neuron [20-21].

The PKA inhibitor KT-5720 is used as a research tool in snail neurons and

Aplysia neurons at concentrations of around 10 μM [15, 35-37]. 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 [28]. Recently, Ro 31-8220 (1-10 μM) [28] has been used in snail neurons [38] and *Aplysia* neurons [33]. In our previous work, pretreatment with KT-5720 (10 μM) and Ro 31-8220 (20 μM) prevented the induction of action potential bursts elicited by methamphetamine (METH) and 3,4-methylenedioxymethamphetamine (MDMA), respectively [20]. In the present study, the action potential bursts elicited by paeonol (500 μM) were not affected by KT-5720 (10 μM; Figure 3) or Ro 31-8220 (20 μM; 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 californa* [39], *Euhadra peliomphala* [40] and *Drosophila* [41]. To understand the mechanism underlying paeonol-elicited potential bursts, the effects of paeonol on ionic currents were tested at a concentration of 500 μM. As shown in Figure 4, paeonol had no remarkable effects on the total inward currents. ~~However, paeonol at 500 μM appears to decrease steady-state outward currents of the RP4 neuron (Figure 6). We therefore focused on the effects of paeonol~~

~~on outward 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 (see our previous study [17]). These findings are consistent with other research involving snail or *Aplysia* neurons [23, 42-43].

The measurement of the I_A followed Thompson's method [23]. The currents were elicited by 200 msec test potentials of -60 mV, -50, -40 and -30 mV from a holding potential of -100 mV. We found that at 20 minutes after paeonol (500 μM and 1.5 mM) application, 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 mM) for 20 minutes. Notably, perfusion with 4-AP (5 mM) elicited no bursts of action potentials in the RP4 neuron (Figure 7B). The finding is consistent with that of our previous study [17]. These results suggest that the effects of paeonol on the I_A current may not directly relate 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 Na^+-free and Co^{2+}-substituted Ca^{2+}-free solution [44]. 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 the outward current was significantly reduced after perfusion with TEA (50 mM). The sensitivity of the outward current to TEA indicated that this is a type of I_{KD}. Notably, a sustained residual current was

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insensitive to TEA[19].

We also sought to determine whether paeonol affects the I_{KD}. Our results indicate that 500 μM paeonol 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 mM) (Figure 6). This 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. Thus, paeonol may have more potency in inhibiting the potassium currents in this preparation.

To further test whether the inhibition of the I_{KD} is involved in the generation of action potential bursts, we examined the effects of TEA on the generation of action potential bursts. As in our previous work, we found that TEA elicited action potential bursts at a concentration of 50 mM (Figure 7D), but not at 5 mM (Figure 8B) [17]. In the present study, paeonol (150 μM) alone failed to elicit action potential bursts (Figure 1B), whereas co-application with TEA (5 mM) did elicit action potential bursts (Figure 8C). These data suggest that inhibition of the 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

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excitability[45]. 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 (p.o.), reduced oxidative stress, cognitive impairment and neurotoxicity induced by D-galactose (D-gal) at doses of 50, 100 mg/kg (p.o.) in mice [12, 46]. Paeonol inhibited carrageenan-evoked thermal hyperalgesia at doses of 30 and 100 mg/ kg (i.p.) in rats [3]. In mice, maximum plasma levels (Cmax) were around 16.3 μM after oral paeonol administration at a dose of 20 mg/kg [47].

In vitro studies used varying concentrations. Paeonol did not show any cytotoxicity against normal human endothelial cells, up to a concentration of 500 μg/ml (around 3 mM) by a trypan blue dye exclusion test, and significantly inhibited proliferation of basic fibroblast growth factor (bFGF)-stimulated human umbilical vein endothelial cells (HUVECs) at the concentration range of 125-500 μg/ml (around 750 μM - 3 mM) [48]. Paeonol at concentratons from 24 μM to 1.5 mM inhibited cell proliferation in HT-29 cells [49] and protected rat neurons from oxygen–glucose

deprivation-induced injury at 0.2-5μM [11]. In addition, paeonol (15.63 - 62.5 mg/L) used in combination with cisplatin had a significantly synergistic growth-inhibitory and apoptosis-inducing effect on 2 human hepatoma cell lines [50] .

As to what concentration of paeonol is required to affect ionic function in excitable tissue, paeonol at concentrations of 160 μM and 640 μM decreased the action potential upstroke phase in guinea pig ventricular cells [10]. At concentrations ranging from 25 μg/ml to 400 μg/ml (150 μM -2.4 mM), paeonol inhibited the delayed outward K^+ current and, to a lesser extent, the I_{Na} in NG108-15 cells [51]. The data from our present study indicate that paeonol at a concentration of 500 μM or higher reversibly elicits action potential bursts of the central RP4 neuron. The concentration used in the present study may be higher than that of 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 (between seizures) 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[52-54]. Research has revealed that convulsants such as pentylenetetrazol (PTZ) induce bursts of action potentials in snail central neurons [40, 55-56], a response that closely resembles PTZ-induced PDS in

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cerebral cortical neurons of mammals [57]. In our previous studies, CNS stimulants such as d-amphetamine, cocaine and methamphetamine were found to elicit *in vitro* action potential bursts in the central RP4 neuron of the African snail, *Achatina fulica* Ferussac [17, 19-20]. This study demonstrated that paeonol at 500 μM induced bursts of action potentials in neurons. These results suggest that paeonol at high doses may have a pro-epileptic side effect. Therefore, special attention is needed when the Moutan cortex of *Paeonia suffruticosa* Andrews (MC) is combined with CNS stimulants.

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}.

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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: revising 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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Figure Legends

Fig. 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 minutes after administration of paeonol (150, 500 and 1500 μM), respectively. E1: Membrane potentials measured at 30 minutes after washing off 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 μM, but were reversibly elicited at concentrations of 500 μM and 1.5 mM.

Fig. 2. Effects of Co^{2+}-substituted Ca^{2+}-free solution on paeonol (500 μM)-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 a RP4 neuron in normal physiological solution. A2: The potentials at 30 minutes after perfusion with Co^{2+}-substituted Ca^{2+}-free solution. B1: Spontaneous action potentials recorded from a RP4 neuron in normal physiological

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solution. B2: Membrane potentials recorded at 20 minutes after perfusion with Co^{2+}-substituted Ca^{2+}-free solution containing paeonol (500 μM).

Fig. 3. Effects of KT-5720 (10 μM; A-E) and Ro 31-8220 (20 μM; 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 from another. A and F: Controls; spontaneous action potentials. B and G: The potentials at 40 minutes after perfusion with KT-5720 (10 μM) and Ro 31-8220 (20 μM), respectively. C and H: The potentials at 20 minutes after further incubation of the neuron with paeonol (500 μM) plus KT-5720 (10 μM) and paeonol (500 μM) plus Ro 31-8220 (20 μM), respectively. . D and I: The potentials at 30 minutes after washing off from C and H, respectively. E and J: The potentials at 20 minutes after incubation of the neuron with paeonol (500 μM) 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 or Ro 31-8220 (20 μM).

Fig. 4. A: Effects of paeonol on 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-msec-long durations in normal

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physiological solution. A1: Control; total inward and outward currents recorded in normal physiological solution. A2: Total inward and outward currents recorded at 20 minutes after incubation with paeonol (500 μM). A3: Voltage step commands. B: Current-voltage relationships of the peak total inward currents before (●), and at 20 minutes after (○), paeonol (500 μM) application. Note that paeonol (500 μM) did not decrease the total inward currents.

Fig. 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 minutes after paeonol (150 μM), paeonol (500 μM) and paeonol (1.5 mM) administration, respectively. E: I_A currents recorded at 30 minutes after washing off from D. F: I_A currents recorded at 20 minutes after 4-AP (5 mM) administration from E. G: Current-voltage relationships of the I_A currents before (●) and after paeonol administration at 500 μM (○) and 1.5 mM (■), respectively. H: Voltage step commands. Note that paeonol (500 μM and 1.5 mM) significantly decreased the I_A at 20 minutes after treatment (*: $p<0.05$ versus control, n=3-4).

Fig. 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

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(50 mM). A and B were recorded from two different RP4 neurons. Currents were elicited by 500-msec-long command steps from holding potentials of -60 mV to test potentials (i.e. 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 minutes after administration of paeonol 150 μM, 500 μM and 1.5 mM, respectively. B1: Steady-state outward currents of the RP4 neuron in TEA (50 mM)-containing Na^+-free and Co^{2+}-substituted Ca^{2+}-free saline. B2: 20 minutes after administration of paeonol (500 μM), from B1. C1 and C2: Effects of paeonol (500 μM and 1.5 mM) on the current-voltage relationships of the steady-state outward currents measured at 500 msec 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 μM) application. The open circle (○) in C1 and closed square (■) in C2 represent the I-V relationship at 20 minutes after paeonol (500 μM and 1.5 mM) application, respectively (*: $p<0.05$ versus control, n=5). C3: Effects of paeonol (500 μM) on the current-voltage relationships of the steady-state outward currents measured at 500 msec of the RP4 neuron in TEA (50 mM)-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 minutes

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after paeonol (500 μM) application, respectively. However, when neurons were pretreated with TEA, paeonol was less effective at decreasing the outward current.

Fig. 7. Effects of 4-AP (5 mM), TEA (50 mM) and charybdotoxin (250 nM) 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 minutes after perfusion with 4-AP (5 mM), TEA (50 mM) and charybdotoxin (250 nM), respectively. The upper left-hand horizontal bar indicates the membrane potential at 0 mV. Note that action potential bursts were not elicited by 4-AP (5 mM) or charybdotoxin (250 nM), but they were by TEA (50 mM).

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

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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.

Variable		N	RMP (mV)	Amplitude of action potential (mV)	Frequency of single spikes (pulses/min)	Frequency of bursts (bursts/min)	Number of action potential /burst
Paeonol	Control	7	-57.7±1.8	85.1±3.3	50.1±11.2		
	Paeonol (150 μM)	4	-58.5±2.7	86.1±6.9	47.3±11.8		
	Paeonol (500 μM)	6	-55.1±1.4	80.8±5.1		8.1±1.6	5.0±0.6
	Paeonol (1.5 mM)	3	-59.3±2.3	83.3±9.6		5.0±1.5	8.7±1.7 [a]
Ro 31-8220 +Paeonol	Control	5	-57.0±1.1	96.9±2.1	28.5±5.0		
	Ro 31-8220 (20 μM)	4	-57.7±1.2	98.8±2.2	30.0±6.7		
	Ro 31-8220 (10 μM) +Paeonol (500 μM)	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 μM)	3	-61.3±1.2	97.8±4.3	30.7±3.8		
	KT-5720 (10 μM) + Paeonol (500 μM)	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 [b]	22.7±5.4		
	Paeonol (500 μM) in Ca^{2+}-free	3	-58.3±1.5	73.5±5.3 [b]		2.7±1.2 [b]	17.3±5.3 [b]

Paeonol+TEA	Control	3	-59.3±3.3	82.3±5.2	51.3±5.3		
	TEA (5 mM)	4	-57.5±1.6	86.0±3.2	29.5±3.0 [b]		
	Paeonol (150 μM) + TEA (5 mM)	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 mM)	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 mM)	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 nM)	3	-57.6±1.2	88.7±3.2	39.3±1.5		

Values are expressed as the mean ± S.E.M. (n being the number of neurons tested).

a: Statistically significant compared with the data of paeonol (500 μM)-elicited action potential bursts ($p < 0.05$).

b : Statistically significant compared with the data in physiological solution (control) ($p < 0.05$).

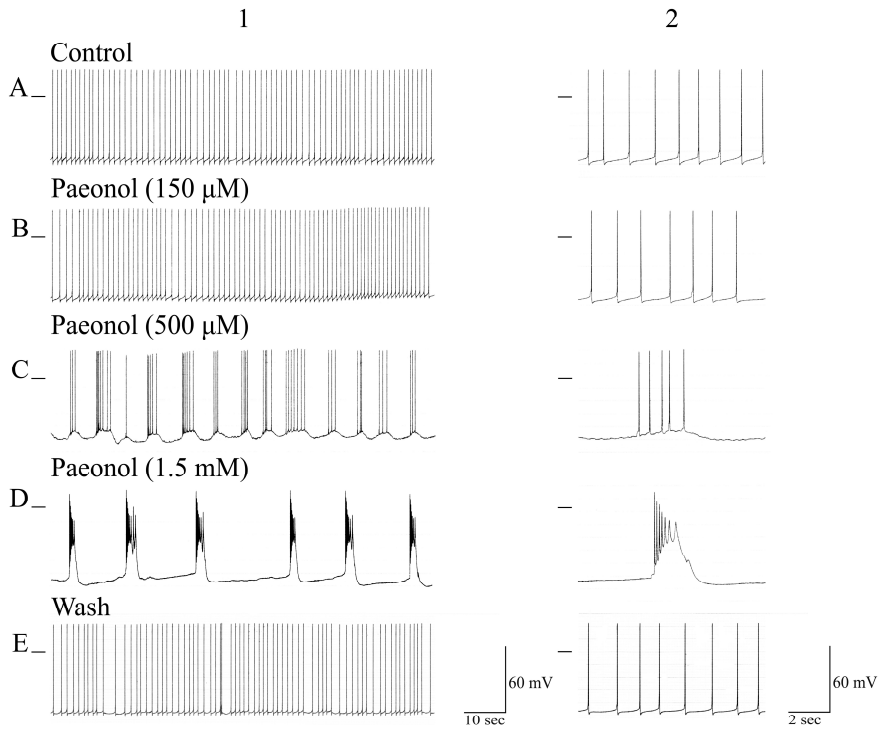

Fig.1
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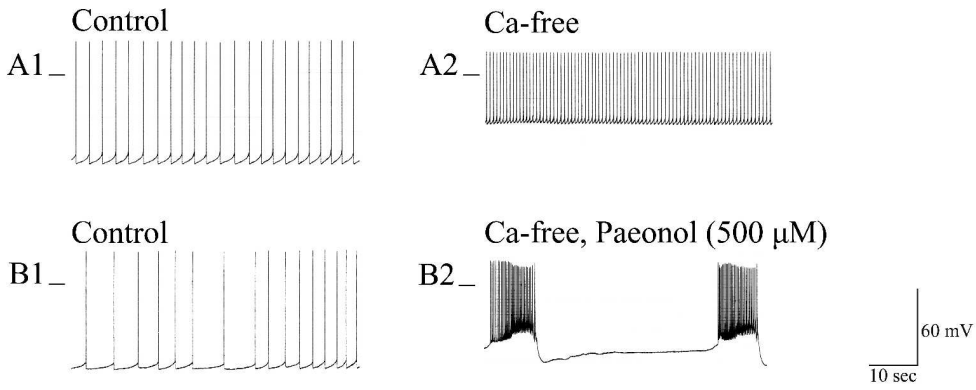


Fig. 2
333x149mm (600 x 600 DPI)

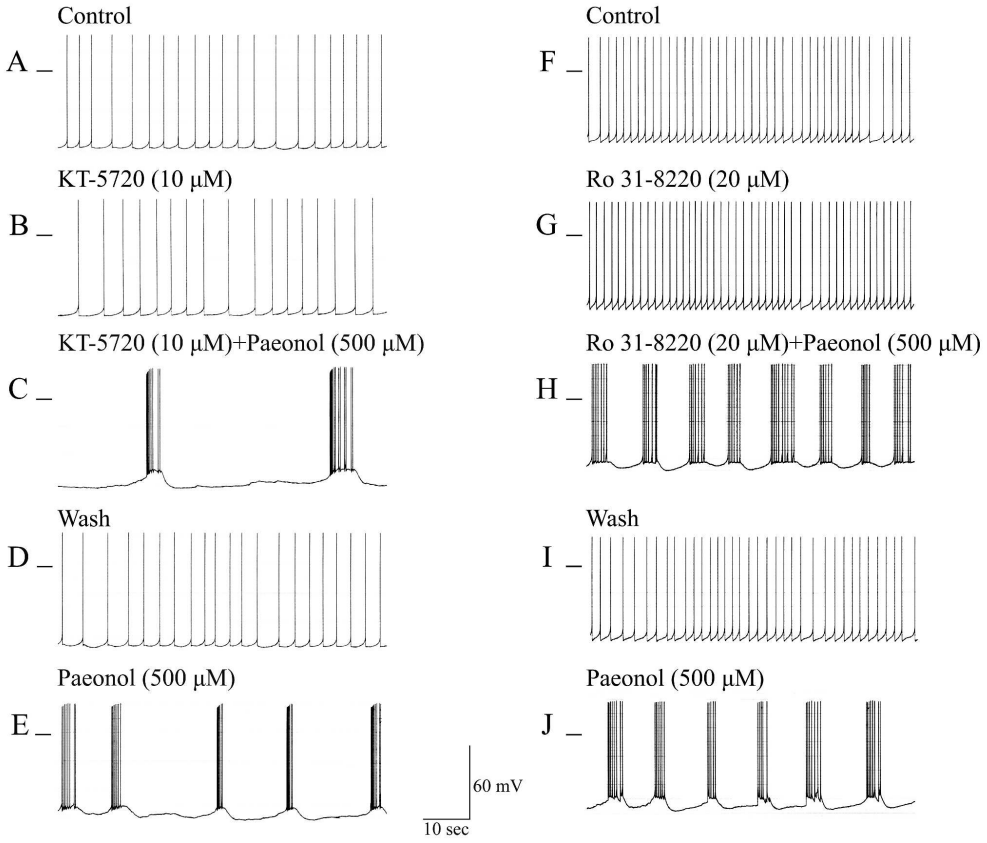


Fig3
347x293mm (300 x 300 DPI)

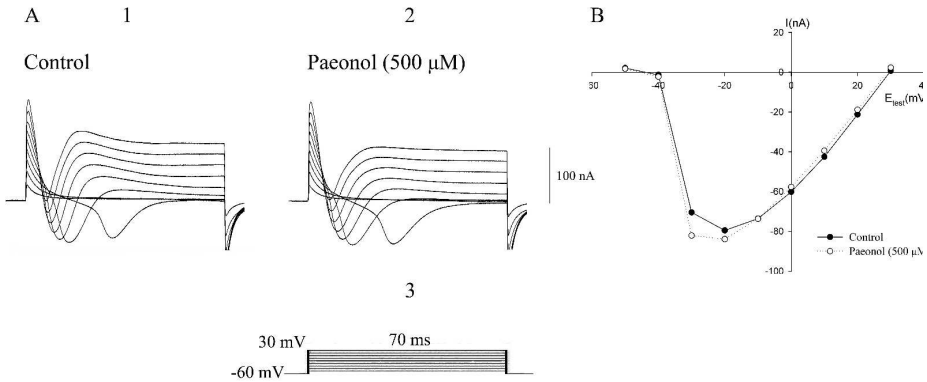
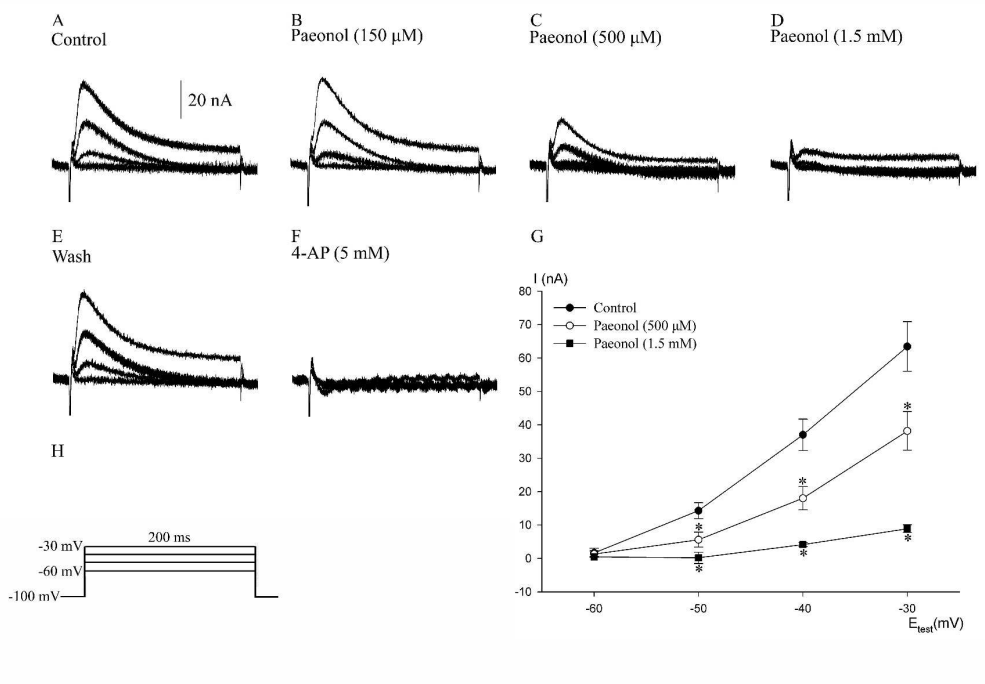
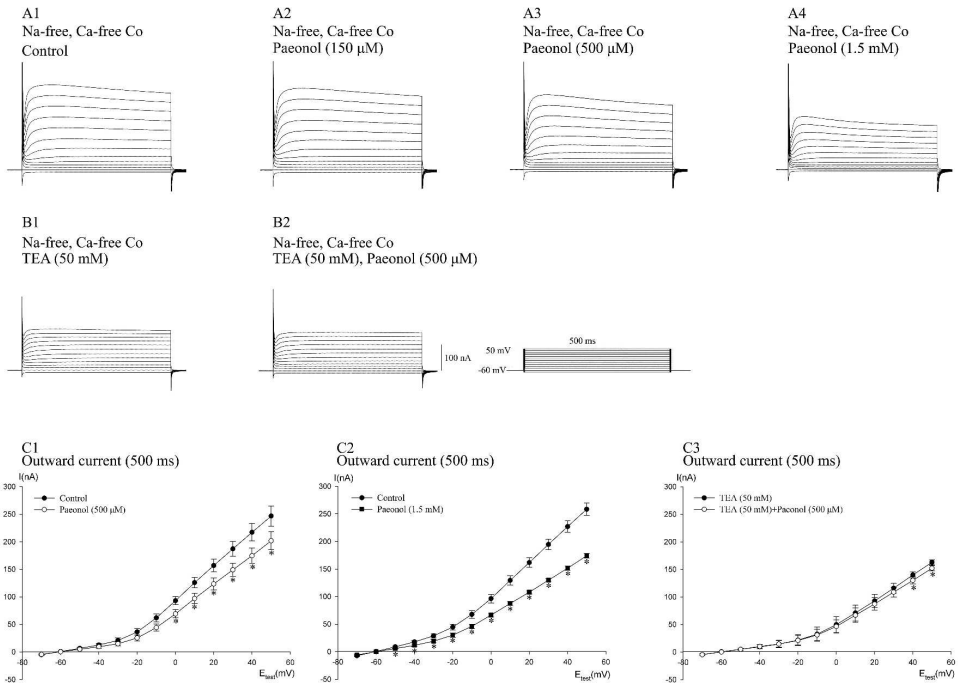


Fig. 4
489x248mm (450 x 450 DPI)



584x403mm (300 x 300 DPI)



700x526mm (200 x 200 DPI)

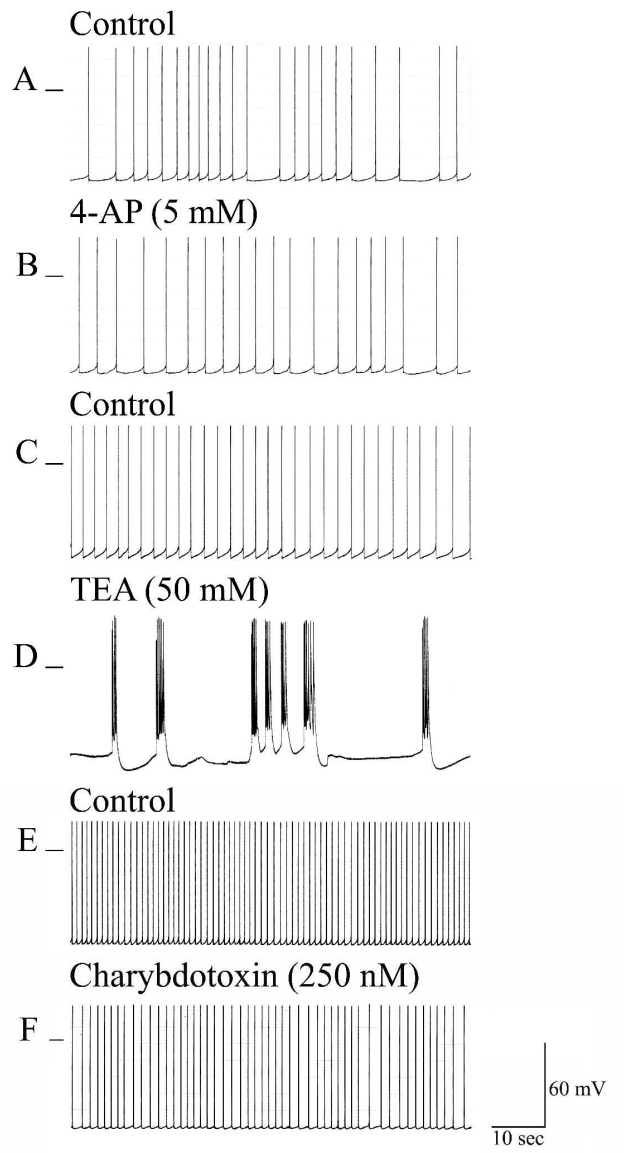


Fig7
190x363mm (600 x 600 DPI)

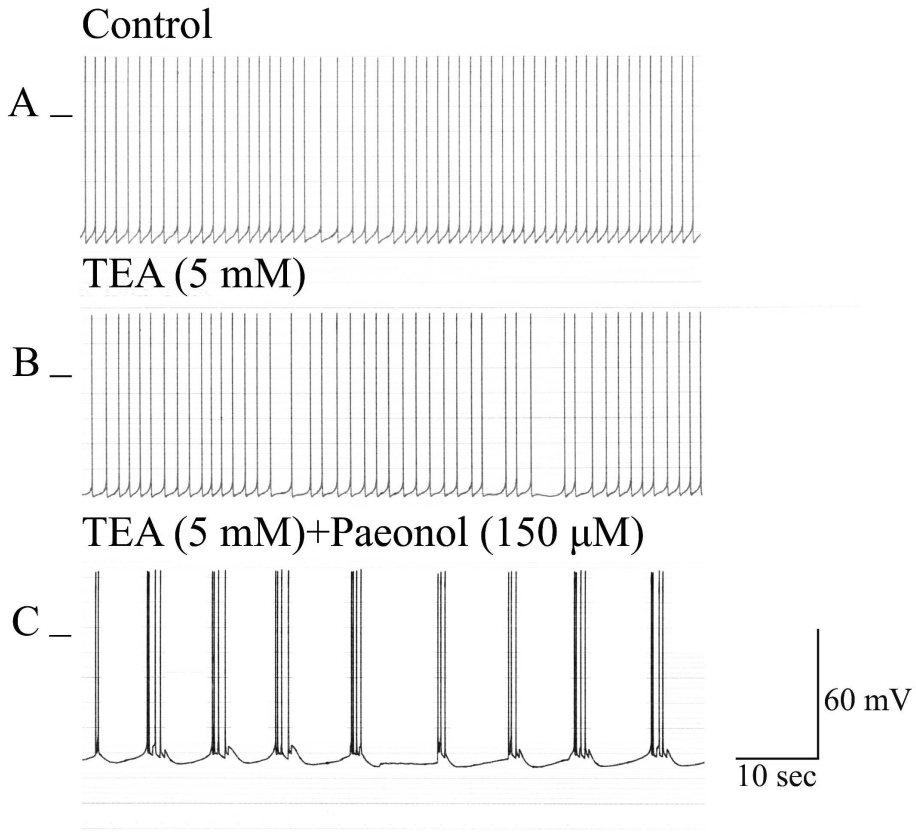


Fig. 8
197x165mm (600 x 600 DPI)