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Action Potential Bursts in Central Snail Neurons Elicited by Procaine: Roles of Ionic Currents

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Abstract

The role of ionic currents on procaine-elicited action potential bursts was studied in an identifiable RP1 neuron of the African snail, *Achatina fulica* Ferussac, using the two-electrode voltage clamp method. The RP1 neuron generated spontaneous action potentials and bath application of procaine at 10 mM reversibly elicited action potential bursts in a concentration-dependent manner. Voltage clamp studies revealed that procaine at 10 mM decreased [1] the Ca^{2+} current, [2] the Na^+ current, [3] the delayed rectifying K^+ current I_{KD}, and [4] the fast-inactivating K^+ current (I_A). Action potential bursts were not elicited by 4-aminopyridine (4-AP), an inhibitor of I_A, whereas they were seen after application of tetraethylammonium chloride (TEA), a blocker of the $I_{K(Ca)}$ and I_{KD} currents, and tacrine, an inhibitor of I_{KD}. Pretreatment with U73122, a phospholipase C inhibitor, blocked the action potential bursts elicited by procaine. U73122 did not affect the I_{KD} of the RP1 neuron; however, U73122 decreased the inhibitory effect of procaine on the I_{KD}. Tacrine decreased the TEA-sensitive I_{KD} of RP1 neuron but did not significantly affect the I_A. Tacrine also successfully induced action potential bursts in the RP1 neuron. It is concluded that the inhibition on the I_{KD} is responsible for the generation of action potential bursts in the central snail RP1 neuron. Further, phospholipase C activity is involved in the procaine-elicited I_{KD} inhibition and action potential bursts.

Key Words: procaine, snail, central neuron, bursts of action potentials, Ca^{2+} current, Na^+ current, phospholipase C, fast-inactivating K^+ current, delayed rectifying K^+ current, ionic currents

Introduction

Procaine is a widely used local anesthetic agent for infiltration, conduction and spinal anesthesia (38). Local anesthetics (LAs), including procaine, can cause CNS and cardiovascular toxicity if their plasma concentrations are increased by accidental intravenous injection or by an absolute overdose (30). LAs-induced convulsion is a common complication in regional anesthesia (16, 28, 38). Scant information exists as to the mechanisms underlying anesthetics-induced seizures. Intravenous injections of procaine at low doses have been shown to depress central nervous system functions and have been recommended for treatment of status epileptics. However, at higher doses, procaine can precipitate seizures in animals and man. The paradoxical activation of central synapses has been attributed to a selective conduction block of inhibitory fibers (5). Despite many investigations, the mechanisms underlying the toxic consequences of

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procaine-induced convulsion remain unclear.

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 (21, 25, 49). In mammals, it is difficult to investigate specific neurons and synapses because of the numerous neurons and glial cells. In contrast, the neurons in ganglia can be identified for investigations into drug-related effects on the same neuron (33). Snail ganglia contain many identifiable neurotransmitters and receptors, and their neurons are used for biological studies (8, 10, 19, 26, 34, 49). Several studies have shown that convulsants, such as pentylenetetrazol, induce bursts of action potentials in snail central neurons (14, 17, 31, 32, 35, 36, 41, 43, 44). This response strongly resembles the pentylenetetrazol-induced paroxysmal depolarizing shift (PDS) seen in cerebral cortical neurons of mammals (42).

Our previous study on the central neuron of the giant African snail (*Achatina fulica* Ferussac) revealed that extracellular application of procaine at 10 mM reversibly elicited bursts of action potentials in the neuron, resembling the pentylenetetrazol-induced paroxysmal depolarizing shift (PDS) seen in cerebral cortical neurons of mammals (11, 25). However, the role of ionic currents on procaine-elicited action potential bursts remained unclear.

The present study aimed to elucidate the effects of procaine on membrane ionic currents during burst activity using the conventional two-electrode voltage clamp technique. Our results indicate that procaine decreased [1] the Ca^{2+} current, [2] the Na^+ current, [3] the delayed rectifying K^+ current (I_{KD}), and [4] the fast-inactivating K^+ current (I_A) in the central snail RP1 neuron. However, the inhibition on the I_{KD} is responsible for the generation of action potential bursts. Further, the phospholipase C inhibitor U73122 decreases the procaine-elicited I_{KD} inhibition.

Materials and Methods

Experiments were performed on identified central RP1 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 (12-14, 24, 25, 51).

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 M potassium chloride (KCl). The experimental chamber was perfused with a control solution, *i.e.* 85 mM NaCl, 4 mM KCl, 8 mM $CaCl_2$, 7 mM $MgCl_2$, 10 mM Tris-HCl, at pH 7.6 and at the 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 (4).

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 3 M KCl.

For measuring the Na^+ current and the Ca^{2+} current, both potential recording and current injection electrodes were filled with 3 M CsCl. All potentials and currents were recorded on tape *via* a digitalizing unit (Digidata 1200, Axon Instruments, Foster City, CA, USA) and analyzed using the pCLAMP software. Ca^{2+} and Na^+ currents were measured using the method described by Adams and Gage (2, 3, 47, 48). For measuring the Ca^{2+} current, the K^+ currents of the neuron were blocked with 4-aminopyridine (4-AP) at 5 mM, tetraethylammonium (TEA) at 50 mM and CsCl at 5 mM. The Na^+ current of the neuron was removed by using Na^+-free solution. The Ca^{2+} currents were elicited by 150 ms command steps from holding potentials of -60 mV to test potentials ranging from -50 to +40 mV, at intervals of 10 mV.

For measuring the Na^+ current, the K^+ currents of the neuron were blocked with 4-AP at 5 mM, TEA at 50 mM and CsCl at 5 mM. Both potential recording and current injection electrodes were filled with 3 M CsCl. The Ca^{2+} current of the neuron was removed with Co^{2+}-substituted Ca^{2+}-free solution. The Na^+ currents were elicited by 70 ms command steps from holding potentials of -60 mV to test potentials ranging from -50 to +70 mV, at intervals of 10 mV.

For measuring the I_A, a Na^+-free solution was used. The currents were elicited by 200 ms test potentials of -60, -50, -40 and -30 mV, from a holding potential of -100 mV (50). For measuring the steady state outward current, Na^+-free and Ca^{2+}-free solutions were used to remove Ca^{2+} currents, Na^+ currents and the Ca^{2+}-activated K^+ current ($I_{K(Ca)}$). The currents of the RP1 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. Both potential recording and current injection electrodes were filled with 3 M KCl to measure both types of K^+ current. In the later stages of this work, some of the experiments used a P/N leak subtraction subpulse routine, as supplied by pCLAMP software.

For measuring Na^+, Ca^{2+} and K^+ currents, the ganglia were perfused with the following solutions:

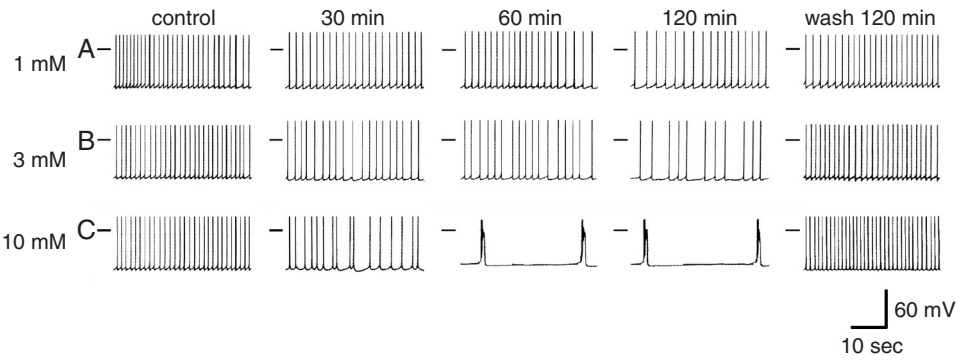

Fig. 1. Effects of procaine on the central RP1 neuron of snails. A, B and C controls represent RP1 neuron potentials. Potentials are also shown in A, B and C after 30, 60 and 120 min of application of procaine at 1, 3 and 10 mM, respectively. Finally, potentials are shown in A, B and C after the procaine preparations were washed off with normal saline for 120 min. The horizontal bar on the top left side is the membrane potential at 0 mV.

(a) physiological solution, (b) Na^+-free solution: equimolar amounts (at 85 mM) of Tris were added to replace Na^+ ions, (c) Co^{2+}-substituted Ca^{2+}-free solution: equimolar amounts (at 8 mM) of Co^{2+} were added to replace Ca^{2+} ions, (d) Co^{2+}-substituted Ca^{2+}-free and Na^+-free solution: equimolar amounts of Tris (at 85 mM) and Co^{2+} (at 8 mM) were added to replace Na^+ and Ca^{2+} ions, respectively. TEA-containing solution was prepared by replacing equimolar quantities of NaCl or Tris-Cl with TEA (47, 48).

Procaine, 4-aminopyridine, TEA and U73122, (1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl) amino]hexyl]-1H-pyrrole-2,5-dione) were purchased from the Sigma Chemical Company (St. Louis, MO, USA). Tacrine was purchased from Tocris Cookson (Bristol, UK). All stocks of the drugs were made with double-distilled water except for U73122, which was prepared in dimethyl sulfoxide (DMSO). The presence of DMSO (≤ 0.1%) alone did not affect the RMPs, amplitude or frequency of the spontaneous firing of action potentials in the RP1 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 $P < 0.05$.

Results

The Electrical Characteristics of the Identifiable RP1 Neuron

The RMP of the identified RP1 neuron was -60.8 ± 1.3 mV (n = 10, means ± S.E.M.) and the spontaneous firing frequency was about 46.7 ± 3.6 pulses/min (n = 10). The single spikes were regularly spaced from each another. No burst firing of action potentials was observed in control RP1 neurons. The mean amplitude of the spontaneously generated action potentials was 84.9 ± 1.2 mV (n = 10).

Effects of Procaine on the RP1 Neuron

Effects of procaine at 1-10 mM on the spontaneous firing action potentials of the central neuron (RP1) are shown in Fig. 1. Procaine did not alter the RMP or amplitude of the generated action potentials of RP1 neurons at 1-3 mM, but did lower the frequency of the spontaneous firing action potentials. Sixty minutes after extracellular perfusion of procaine at 1 mM and procaine at 3 mM, the RMPs of the generated action potentials of the RP1 neurons were -62.8 ± 0.2 mV (n = 10) and -63.1 ± 0.7 mV (n = 10), respectively. The amplitudes of the generated action potentials of RP1 neurons were 86.8 ± 0.3 mV (n = 10) and 87.1 ± 0.9 mV (n = 10), respectively. Increasing the procaine concentration to 10 mM, the RMP and amplitude of the generated action potentials of RP1 neurons were -58.8 ± 0.9 mV (n = 10) and 79.8 ± 1.3 mV (n = 10). Sixty min after procaine administration at 1 mM and 3 mM, the frequency of the spontaneous firing action potential was decreased from 47.4 ± 2.6 pulses/min (control) to 33.9 ± 2.3 pulses/min in 1 mM ($P < 0.05$, n = 10) and 27.3 ± 0.8 pulses/min in 3 mM ($P < 0.05$, n = 10). It appears that procaine did not alter the action potential firing pattern of the RP1 neuron at concentrations of 1 mM and 3 mM. Procaine (10 mM) elicited bursts of action potentials 60 min after procaine administration, lasting for more than 2 h. The firing pattern changed from regularly spaced single spikes to one in which bursts of 2 to 10 action potentials were separated by a large hyperpolarization of membrane potentials (up to 6 mV) lasting 1 to 30 sec. The frequency of bursts was 5.6 ± 1.9 bursts/min (n = 10).

The bursts of action potentials elicited by pro-

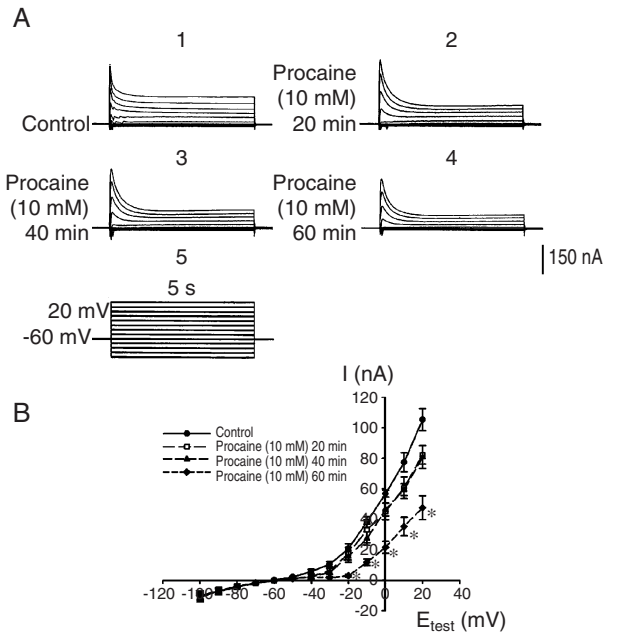

Fig. 2. Effects of procaine on steady-state currents of the RP1 neuron. The currents were elicited by 5-s-long command steps from a holding potential of -60 mV to test potentials of -100 and +20 mV, at intervals of 10 mV. A1: control, steady-state currents recorded in normal physiological saline. A2, A3 and A4: steady-state currents recorded at 20 min, 40 min and 60 min after application of procaine at 10 mM, respectively. A5: voltage step commands. B: Current-voltage relationships of the steady-state currents before (●) and after 20 (□), 40 (▲) and 60 (◆) min in procaine. *: $P < 0.05$ versus control, paired *t*-test, n = 8.

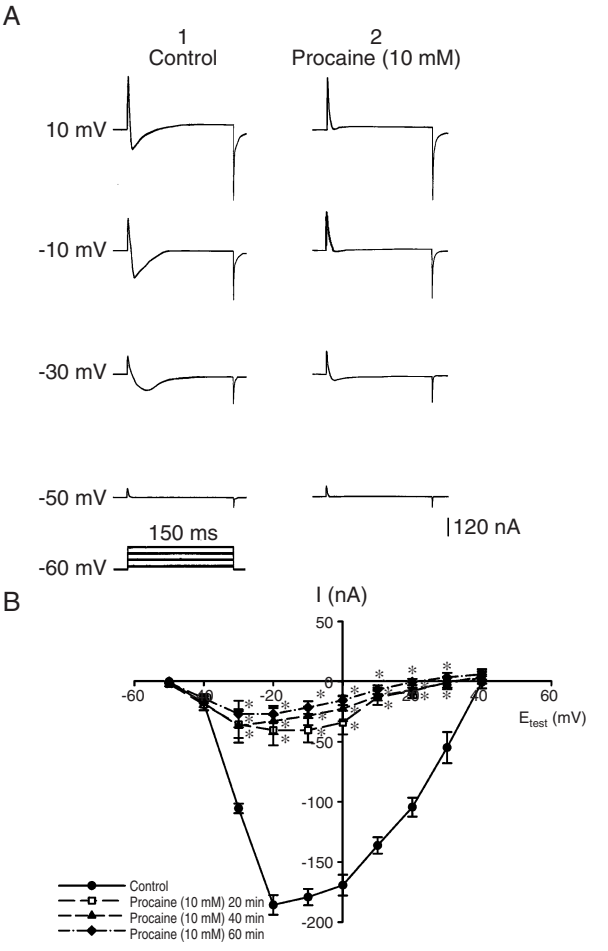

Fig. 3. Effects of procaine on Ca^{2+} currents of the RP1 neuron. A1: Control Ca^{2+} currents in Na^{+}-free saline containing 5 mM 4-AP, 50 mM TEA and 5 mM CsCl. A2: Ca^{2+} currents recorded at 60 min after procaine application at 10 mM. B: Current-voltage relationships of the peak Ca^{2+} currents before (●) and after 20 (□), 40 (▲) and 60 (◆) min in procaine. *: $P < 0.05$ versus control, paired *t*-test, n = 6.

caine recovered to those of control levels after continuous washing off procaine with normal saline for 120 min (Fig. 1).

Effects of Procaine on the Steady-State Currents of the RP1 Neuron

The effects of procaine on the steady-state currents of the RP1 neuron are shown in Fig. 2. The steady-state currents were elicited by stepping from -60 mV to test potentials of -100 to 20 mV, at intervals of 10 mV in normal physiological solution, as shown in Fig. 2A. The steady-state currents were measured at 5 s after voltage stepping and the steady-state I-V relationships are shown in Fig. 2B. Compared with the pre-drug control, the steady-state currents were significantly decreased 60 min after procaine treatment, if the positive values of the test potentials were at voltages higher than -20 mV (decreased by 85.8 ± 1.7%, paired *t*-test, $P < 0.05$, n = 8, stepped to -20 mV). However, no negative slope resistance in steady-state current-voltage (I-V) relationships was revealed in the presence of procaine at 10 mM.

Effects of Procaine on the Ca^{2+} Current and Na^{+} Current

The effects of procaine on the Ca^{2+} current and Na^{+} current are shown in Fig. 3A and 4A, respectively. The I-V prior to and at 20, 40 and 60 min after procaine (10 mM) application is shown in Figs. 3B and 4B, respectively. At 20, 40 and 60 min after 10 mM procaine application, the peak Ca^{2+} current amplitude was decreased at test potentials of -30 to 30 mV (Fig. 3B) and the peak Na^{+} current amplitudes were decreased at test potentials of -40 to 60 mV (Fig. 4B).

Effects of Procaine on the I_A Current

The effects of up to 60-min application of procaine on the I_A currents are shown in Fig. 5. It appears

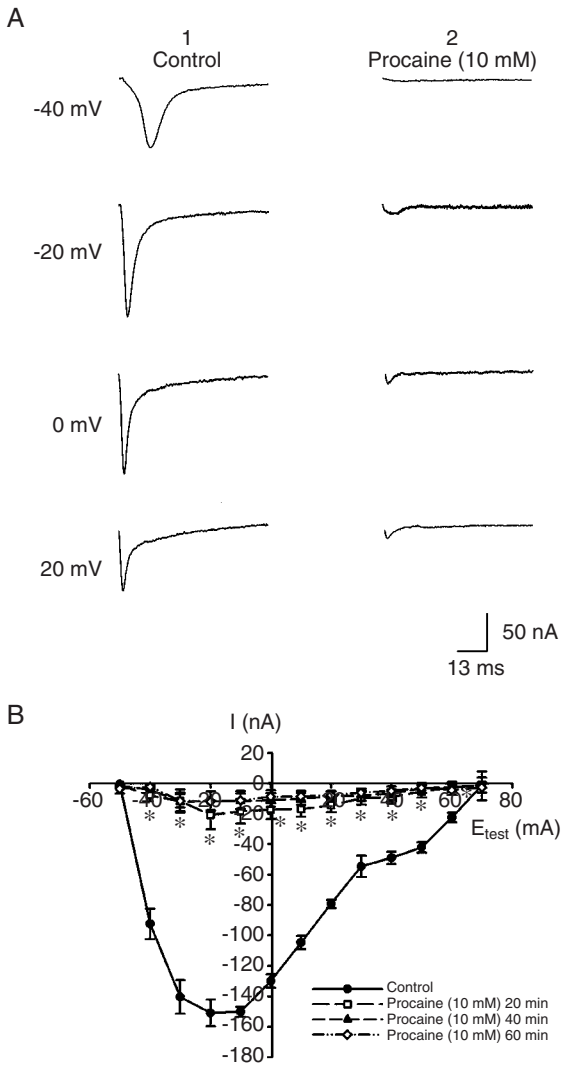

Fig. 4. Effects of procaine on Na^+ currents of the RP1 neuron. A1: Control Na^+ currents in Co^{2+}-substituted Ca^{2+}-free saline containing 5 mM 4-AP, 50 mM TEA and 5 mM CsCl. A2: Na^+ currents recorded at 60 min after procaine application at 10 mM. B: Current-voltage relationships of the peak Na^+ currents before (●) and after 20 (□), 40 (▲) and 60 (◇) min in procaine. The leak and transient capacitance currents of the neuron were subtracted in this figure. *: $P < 0.05$ versus control, paired *t*-test, n = 5.

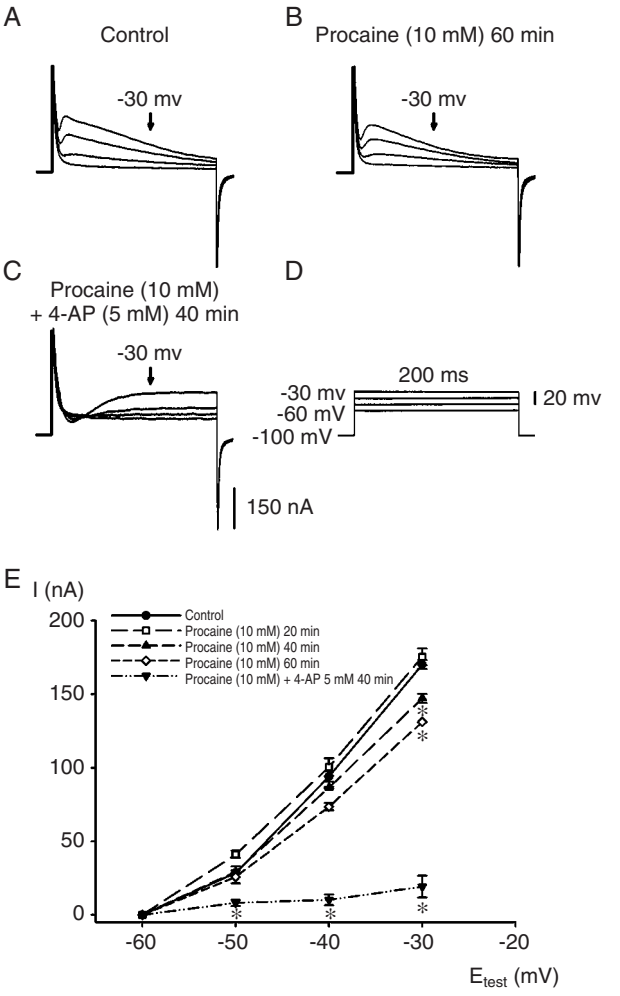

Fig. 5. Effects of procaine (10 mM) on the peak amplitude of the I_A current of the RP1 neuron. A: Control I_A currents recorded in Na^+-free solution. B: I_A currents recorded 60 min after procaine (10 mM) administration from A. C: I_A currents recorded 40 min after 4-AP (5 mM) administration from B. D: The voltage step commands. E: Current-voltage relationships of the I_A currents before (●) and after 20 (□), 40 (▲) and 60 (◇) min in procaine, and at 40 min after procaine and 4-AP (▼). *: $P < 0.05$ versus control, paired *t*-test, n = 5.

that peak I_A current amplitudes were significantly decreased at ≥40 min at test potentials of -30 mV in the presence of procaine; the I_A current was completely abolished if 4-AP at 5 mM was applied to the bath solution for 40 min.

Effects of Procaine on the Steady-State Outward Current in Na^+-Free and Co^{2+}-Substituted Ca^{2+}-Free Solution

To elucidate the components of the steady-state outward current of the RP1 neuron, we tested the effects of removal of calcium ions, TEA and 4-AP on the steady-state outward current. The steady-state outward current of the RP1 neuron was firstly measured with 500-msec-long voltage clamp steps in a Na^+-free solution. An example is shown in Fig. 6. A slow decay of the outward current was observed after applying potential steps exceeding -20 mV (Fig. 6A). If the neuron was further perfused with Na^+-free and Co^{2+}-substituted Ca^{2+}-free solution for 40 min, the outward currents were significantly decreased (Fig. 6B). The sensitivity to extracellular calcium ions suggests a I_{KCa}.

I_A and I_{KD} components in the outward currents were further identified by TEA and 4-AP. The amplitude at 500 msec of the outward current was signi-

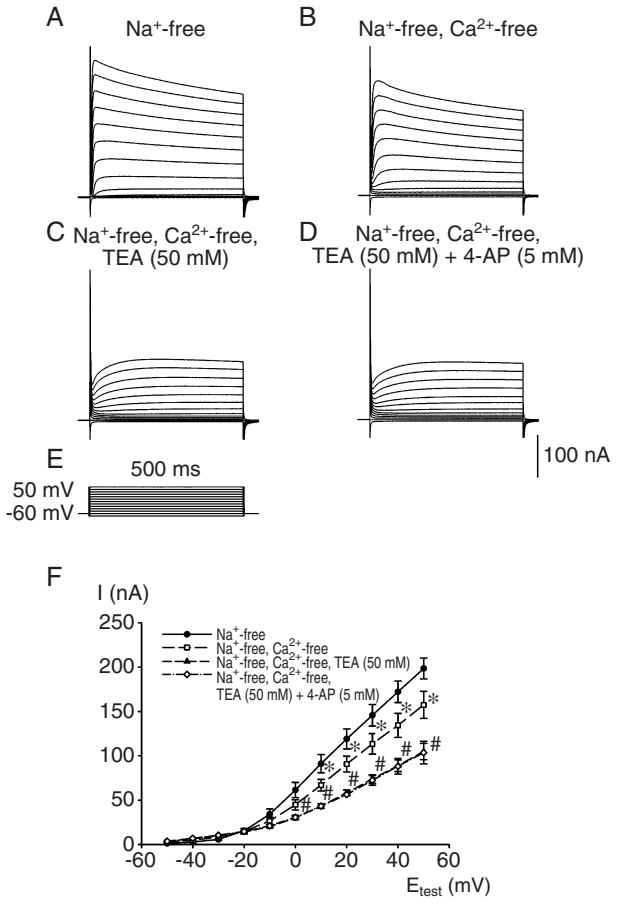

Fig. 6. Effects of removal of sodium ions, calcium ions, TEA and 4-AP on steady-state outward currents of the RP1 neuron. A, B, C and D were recorded from the same RP1 neuron. A: Steady-state outward currents recorded in Na^+-free saline. B: Steady-state outward currents recorded at 40 min after perfusion with Na^+-free and Co^{2+}-substituted Ca^{2+}-free saline from A. C: The currents 40 min after TEA at 50 mM was further incubated from B. D: The currents 40 min after 4-AP at 5 mM was further incubated from C. E: The voltage step commands. F: Current-voltage relationships of the steady-state outward currents in Na^+-free (●), Na^+-free and Ca^{2+}-free saline (□), TEA (▲) in Na^+-free and Ca^{2+}-free saline and TEA with 4-AP (◇) in Na^+-free and Ca^{2+}-free saline, *: $P < 0.05$ versus Na^+-free saline, paired *t*-test, n = 6. #: $P < 0.05$ versus Na^+-free and Co^{2+}-substituted Ca^{2+}-free saline, paired *t*-test, n = 6.

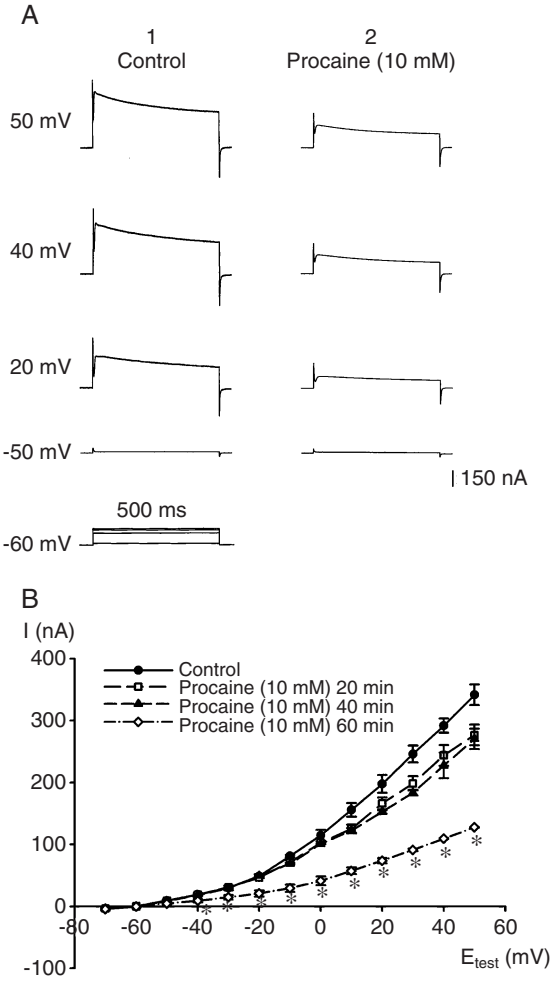

Fig. 7. Effects of procaine on steady-state outward currents of the RP1 neuron. The Ca^{2+} current and Na^+ current were eliminated using Co^{2+}-substituted Ca^{2+}-free saline and Na^+-free saline. The steady-state outward currents in control (A1) and 60 min after incubation of procaine at 10 mM (A2). The lowest trace shows the voltage step commands. (B) Current-voltage relationships of the steady-state outward current before (●) and after 20 (□), 40 (▲) and 60 (◇) min in procaine. *: $P < 0.05$ versus control, paired *t*-test, n = 5.

ficantly reduced when perfused with TEA at 50 mM for 40 min (Fig. 6C). If 4-AP at 5 mM was applied to the bath solution for 40 min, the steady-state outward current remained unchanged (Fig. 6D). The current-voltage relationships of steady-state outward current are shown in Fig. 6F. It appears that removal of calcium ions and TEA decreased the steady-state outward currents. However, treatment with 4-AP did not further decrease the steady-state outward currents. Similarly, when 4-AP at 5 mM was applied to the Na^+-free and Co^{2+}-substituted Ca^{2+}-free solution for 40 min, the amplitude at 500 ms of the outward current was not affected (Figs. 8, B and F). The amplitude at 500 ms of the outward current was significantly reduced when incubated with TEA (50 mM) for 60 min (Figs. 8, C1 and F). The sensitivity of the outward current to TEA indicates that this is a type of I_{KD} current. However, there was a sustained residual TEA-insensitive current.

The effects of procaine on the steady-state outward current and its current-voltage relationships are shown in Figs. 7 and 8. Currents at test potentials in the range of -40 to 50 mV were significantly decreased after 60 min of procaine administration at 10

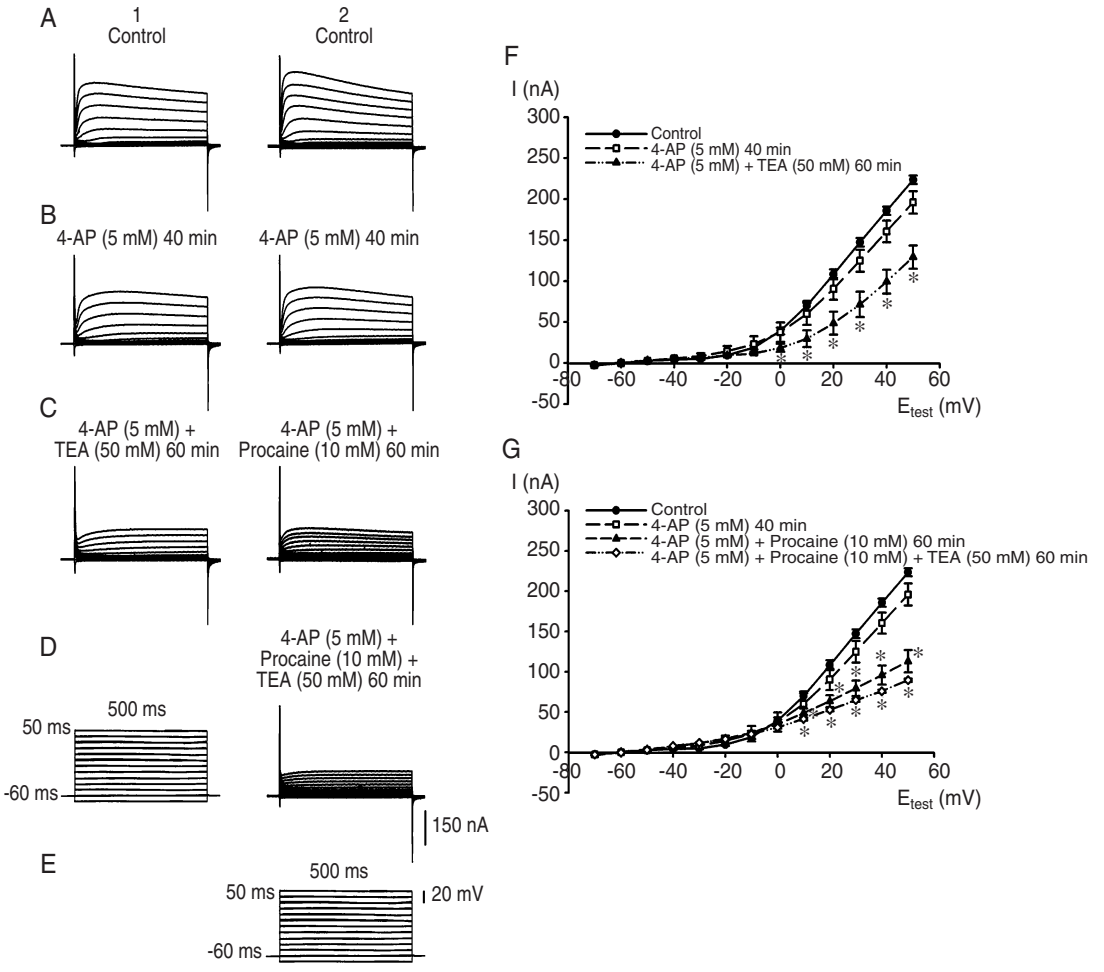

Fig. 8. Effects of 4-AP (5 mM), TEA and procaine on the steady-state outward currents in the RP1 neuron. The Ca^{2+} current and Na^{+} current were eliminated using Co^{2+}-substituted Ca^{2+}-free saline and Na^{+}-free saline. A1, B1 and C1 were recorded from one RP1 neuron, while A2, B2, C2 and D2 were from another. A1 and A2 were control, represent steady-state outward currents in the Na^{+}-free and Co^{2+}-substituted Ca^{2+}-free saline. B1 and B2 represent the steady-state outward current recorded after 40-min incubation with 4-AP at 5 mM. C1 represents the steady-state outward current recorded after 60 min incubation with 4-AP and TEA at 50 mM. C2 represents the steady-state outward current recorded after 60 min incubation with 4-AP and procaine at 10 mM. D2 represents the steady-state outward currents recorded after 60 min incubation with 4-AP, procaine and TEA. D1 and E2 represent the voltage step commands. F and G: I-V relationships of the steady-state outward currents in the RP1 neuron. F: Current-voltage relationships of the steady-state outward current before 4-AP application (●), at 40 min after 4-AP application (□), and at 60 min after 4-AP and TEA application (▲). G: The steady-state outward currents before 4-AP application (●), at 40 min after 4-AP application (□), at 60 min after 4-AP and procaine application (▲), and at 60 min after 4-AP, procaine and TEA application (◇). *: $P < 0.05$ versus control, paired *t*-test, n = 5.

mM (Fig. 7). However, the current was not further decreased by the addition of 50 mM TEA for 60 min, measured at 500 ms (Figs. 8, D2 and G).

Effects of U73122 on Procaine-Elicited Potential Changes on the RP1 Neuron

U73122 is a known phospholipase C inhibitor (15); an example of the effects of U73122 at 10 μM on the procaine-elicited potential changes is shown in Fig. 9. U73122 at 10 μM did not alter the resting membrane potential, amplitude and frequency of the spontaneously generated action potentials of the RP1 neuron (Fig. 9A). Procaine at 10 mM elicited bursts of action potentials 60 min after procaine administration (Fig. 9B). No bursts of action potentials were found in U73122- and procaine-treated preparations tested (Fig. 9C). Compared with the neurons treated with U73122 at 10 μM and neurons treated with U73122 and procaine, U73122 and procaine decreased the frequency of the spontaneously generated action potentials of the RP1 neuron ($P < 0.05$), while it did not alter the resting membrane potential and amplitude of the spontaneously generated action potentials of

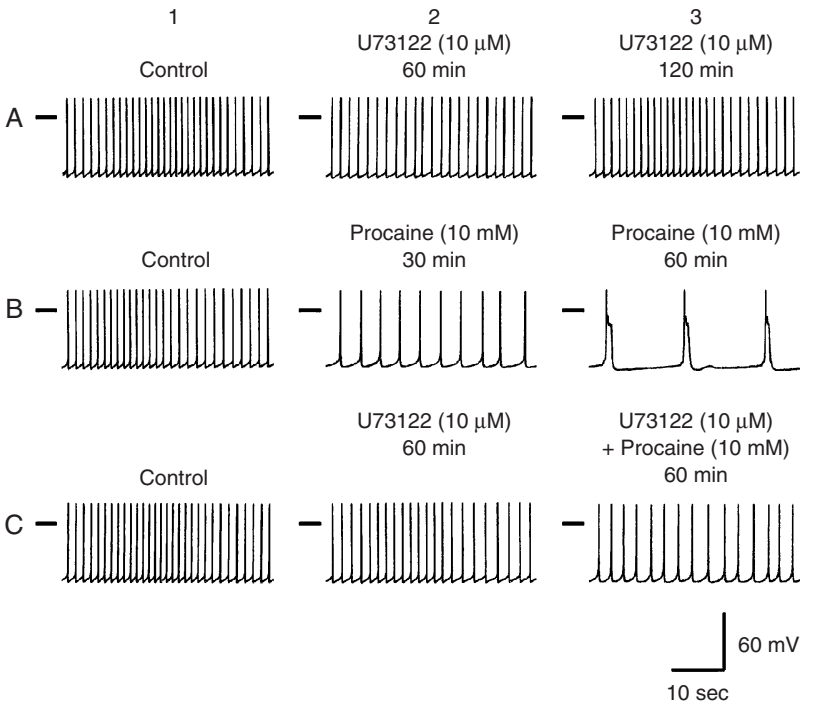

Fig. 9. Effect of U73122 (10 μM) (PLC inhibitor) on procaine-elicited bursts of action potentials. A1, B1 and C1 were control, showing spontaneous firing of action potentials. A2, A3 were the potentials after further addition of U73122 at 10 μM 60 and 120 min from A1, respectively. B2, B3 were the potentials after addition of procaine at 10 mM 30 and 60 min from B1, respectively. C2 and C3 were the potentials before and after addition procaine 60 min in the presence of U73122.

the RP1 neurons.

Effects of U73122 on Procaine-Elicited Changes to the Steady-State Outward Current in Na^+-Free and Co^{2+}-Substituted Ca^{2+}-Free Solution

The effects of U73122 on procaine-elicited changes to the steady-state outward current in Na^+-free and Co^{2+}-substituted Ca^{2+}-free solution are shown in Fig. 10. When the amplitude of the steady-state outward current was measured at 500 ms of the outward current, the amplitude remained unchanged after 60 min of incubation with U73122 at 10 μM, but decreased after 60 min of incubation with procaine at 10 mM alone; the reduction was 39.7 ± 1.0% compared with the control ($P < 0.05$, n = 6, stepped to 50 mV). Notably, treated with U73122 and procaine, the steady-state outward currents in the range of 10-50 mV remained larger than those observed after 60 min of incubation with procaine in the absence of U73122, and the reduction was 18.1 ± 1.3% compared with the control ($P < 0.05$, n = 6, stepped to 50 mV).

Effects of 4-AP, TEA and Tacrine on the Spontaneous Action Potentials of the RP1 Neuron

To elucidate the role of I_{KD}, I_A and $I_{K(Ca)}$ in the generation of the action potential bursts elicited by procaine, we tested the effects of 4-AP, TEA and tacrine on spontaneous action potentials. An example of the effects of 4-AP at 5 mM on the spontaneously generated action potentials is shown in Fig. 11A. Application of 4-aminopyridine (4-AP), an inhibitor of I_A (9), failed to elicit action potential bursts. When 4-AP at 5 mM was administered for a further 60 min, the RMP was -63.1 ± 1.7 mV (n = 4). The frequencies of spontaneously generated action potentials 60 min after the 4-AP application were 30.0 ± 5.4 pulses/min (n = 4; $P < 0.05$ *vs*. the control condition); however, no bursting activity of potentials was observed. The effects of 4-AP were reversible. Sixty minutes after washing off 4-AP, the RMP and the spontaneously generated action potentials of RP1 neurons recovered to those of control levels.

Tetraethylammonium chloride (TEA) blocks the $I_{K(Ca)}$ (27, 29, 52) and the I_{KD} (37). TEA at 50 mM elicited bursts of action potentials in the RP1 neurons. The frequencies of spontaneously generated action potentials at 60 min after TEA administration were 13.0 ± 2.0 bursts/min (n = 4). The effects of TEA on the RP1 neurons were reversible. Sixty min after washing off TEA, the RMP and action potential values recovered to those of control levels. An example of the effects of TEA at 50 mM on the spontaneous firing of action potentials in the RP1 neuron is shown in Fig. 11B.

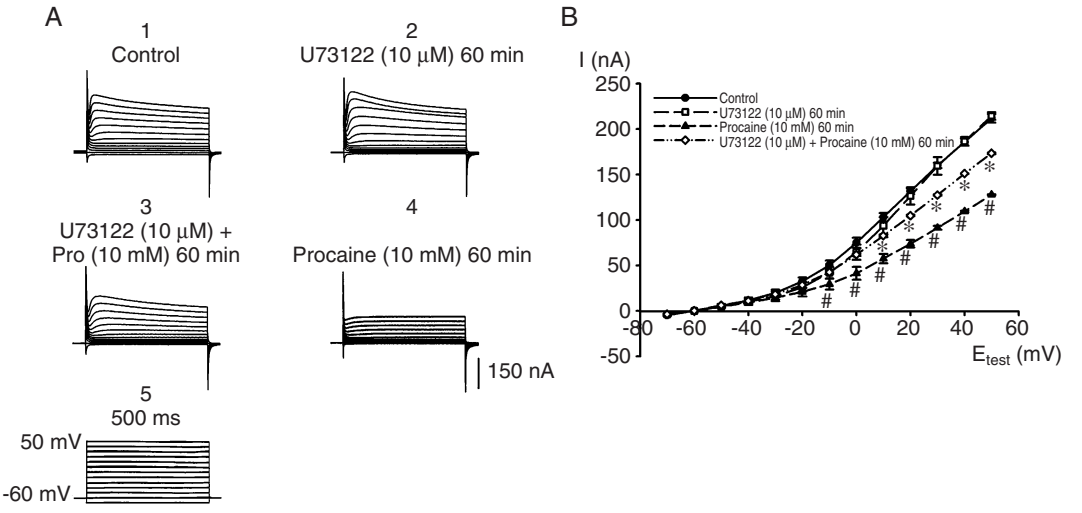

Fig. 10. Effects of U73122 at 10 μM on procaine-elicited steady-state outward current changes in the RP1 neuron. Na^+-free and Co^{2+}-substituted Ca^{2+}-free saline was used to remove the Ca^{2+} current, Na^+ and I_{KCa} currents. A1, A2, A3 and A4 were recorded from the same RP1 neuron. A1: Control, steady-state outward currents in the Na^+-free and Co^{2+}-substituted Ca^{2+}-free saline. A2: Steady-state outward currents recorded at 60 min after incubation with U73122 at 10 μM from A1. A3: Steady-state outward currents recorded at 60 min after incubation with procaine at 10 mM and U73122 from A2. A4: Steady-state outward currents recorded at 60 min after washing off U73122 from A3. A5: Voltage step commands. B: Effects of U73122 and procaine on the I-V relationships of the steady-state outward currents of the RP1 neuron in Co^{2+}-substituted Ca^{2+}-free and Na^+-free solution. Closed circles (●) and open squares (□) represent the steady-state outward current-voltage relationships before and after U73122 application at 60 min, respectively. Closed triangles (▲) and open diamonds (◇) represent the steady-state outward current-voltage relationships after procaine (10 mM) application alone at 60 min and after incubation with U73122 and procaine at 60 min, respectively. #: $P < 0.05$ versus control, paired *t*-test, n = 6. *: $P < 0.05$ versus procaine, paired *t*-test, n = 6.

Tacrine, a centrally acting anticholinesterase agent used to treat Alzheimer's disease (18, 20, 46), reportedly only inhibits I_{KD} without affecting other ionic currents in muscle cells of *Drosophila* (23). The effects of 30- and 60-min incubation with tacrine at 150 μM and at 500 μM are shown in Fig. 11. Action potential bursts were elicited after 30 min incubation with tacrine at 150 μM (n = 4). Increasing the tacrine concentration to 500 μM induced more remarkable bursts of action potentials (Figs. 11, D and E). The frequency of bursts of action potentials in the RP1 neuron after 30 min of incubation in tacrine at 500 μM was 12.5 ± 0.7 bursts/min (n = 4).

Effects of Tacrine on the Steady-State Outward Current in Na^+-Free and Co^{2+}-Substituted Ca^{2+}-Free Solution

The effects of tacrine on the steady-state outward current and its current-voltage relationships are shown in Fig. 12. The steady-state outward currents of the RP1 neuron were measured with 500-ms-long voltage-clamp steps in a Na^+-free and Co^{2+}-substituted Ca^{2+}-free solution. The amplitude of the steady-state outward current measured at 500 ms was decreased by application of tacrine at 150 μM for 40 min (Figs. 12, B and H). The amplitude at 500 ms of the outward current was significantly reduced when incubated with tacrine at 500 μM for 40 min reduced by and the reduction was 36.4 ± 3.6% compared with the control ($P < 0.05$, n = 4, stepped to 50 mV) (Figs. 12, C and I). After the addition of TEA at 50 mM, the steady-state current was further decreased (Fig. 12D).

When the RP1 neuron was perfused in 50 mM TEA-containing Co^{2+}-substituted Ca^{2+}-free and Na^+-free solution, the addition of tacrine at 500 μM did not further decrease the steady-state current (Figs. 12, E, F and J).

Effects of Tacrine on the I_A Current

An example of the effects of tacrine on the I_A current is shown in Fig. 13; the I_A current was completely abolished if 4-AP at 5 mM was applied to the Na^+-free solution for 40 min (Fig. 13A2).

When neurons were perfused for 40 min with tacrine at 150 μM and 500 μM, the peak amplitude of I_A was not affected. Examples of current-voltage relationships are shown in Figs. 13, B and C.

Discussion

In the present study, the effects of procaine on

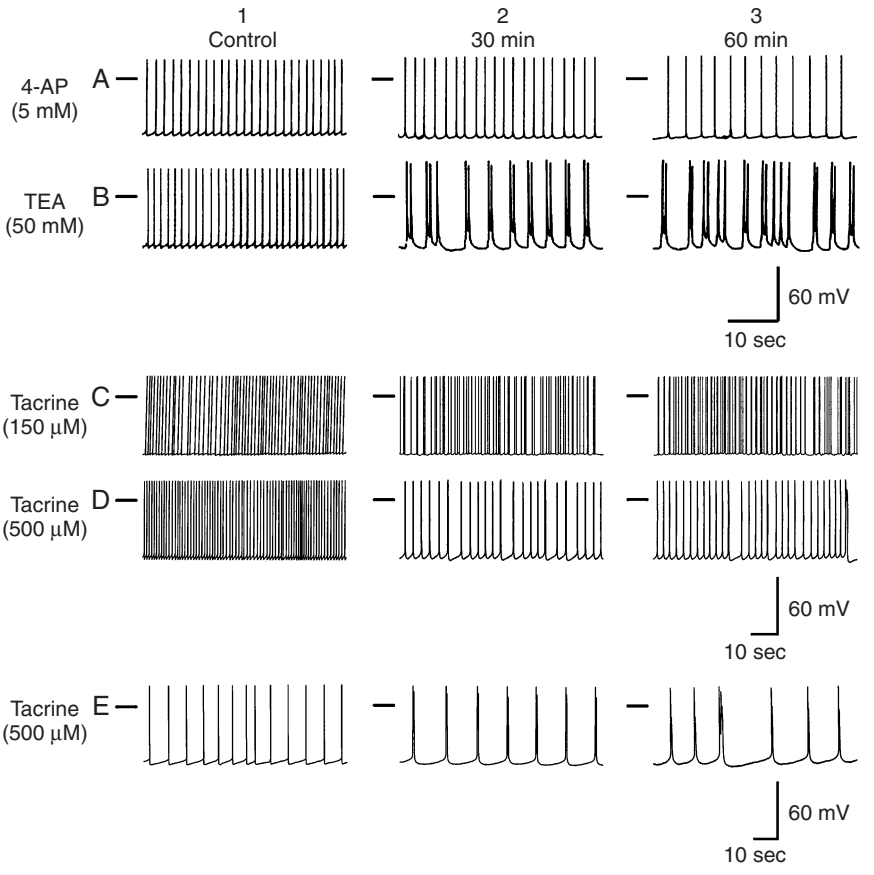

Fig. 11. Effects of 4-AP, TEA and tacrine on the spontaneous action potentials of the RP1 neuron. A1, B1, C1 and D1 represent controls, showing spontaneous firing of action potentials. A2 and A3 represent potentials at 30 and 60 min after application of 4-AP at 5 mM, respectively. B2 and B3 represent potentials at 30 and 60 min after application of TEA at 50 mM, respectively. C2 and C3 represent potentials at 30 and 60 min after application of tacrine at 150 μM, respectively. D2 and D3 represent potentials at 30 and 60 min after application of tacrine at 500 μM, respectively. E1, E2 and E3 represent expanded pictures of D1, D2 and D3, respectively. The horizontal bar at the top left indicates the membrane potential at 0 mV.

the spontaneous action potentials and ionic currents of the RP1 neuron of the African snail were examined. The neuron exhibited spontaneous regular firing of action potentials. No bursts of action potentials were found in control RP1 neurons, whereas extracellular application of procaine at 10 mM for 60 min reversibly elicited bursts of action potential spikes in the RP1 neuron. The results are consistent with those reported previously (25).

Ionic currents play an important role in the firing of action potential bursts, as seen in the neurons of *Aplysia californa* (6, 39, 53), *Euhadra peliomphala* (31, 32, 35, 36) and *Drosophila* (54). We first examined the effects of procaine on the steady-state currents. To understand the mechanism underlying the procaine-elicited potential bursts, the effects of procaine on ionic currents were tested at a concentration of 10 mM. The results revealed that procaine appeared to decrease steady-state currents (Fig. 2) of the RP1 neuron. To further understand the effects of procaine on individual currents, we measured the Na^+, Ca^+ and K^+ currents of the RP1 neuron using the two-electrode voltage clamp method as described in our previous study (12). In the present study, we found that peak Ca^{2+} and Na^+ current amplitudes were significantly decreased at ≥20 min in procaine (Figs. 3B and 4B) It is noted that procaine elicited action potential bursts only at 60-min incubation, but not after 20 min or 40 min of incubation. It is assumed that the effects of procaine on the Ca^{2+} and Na^+ currents may not directly be related to its effect on bursts of action potentials. This contention is further supported by the observation that perfusion with Ca^{2+}-free solution, which abolishes the Ca^{2+} current and the $I_{K(Ca)}$, does not cause any bursts of action potentials in control RP1 neurons (25). In addition, Na^+-free solution does not elicit action potential bursts (25).

With regard to the outward K^+ currents, at least three K^+ currents exist in snail neurons, *i.e.* a I_A, a $I_{K(Ca)}$, and a I_{KD}. TEA and 4-AP are two agents which are most frequently used to study K^+ currents in molluscan neurons; I_A is sensitive to 4-AP, while

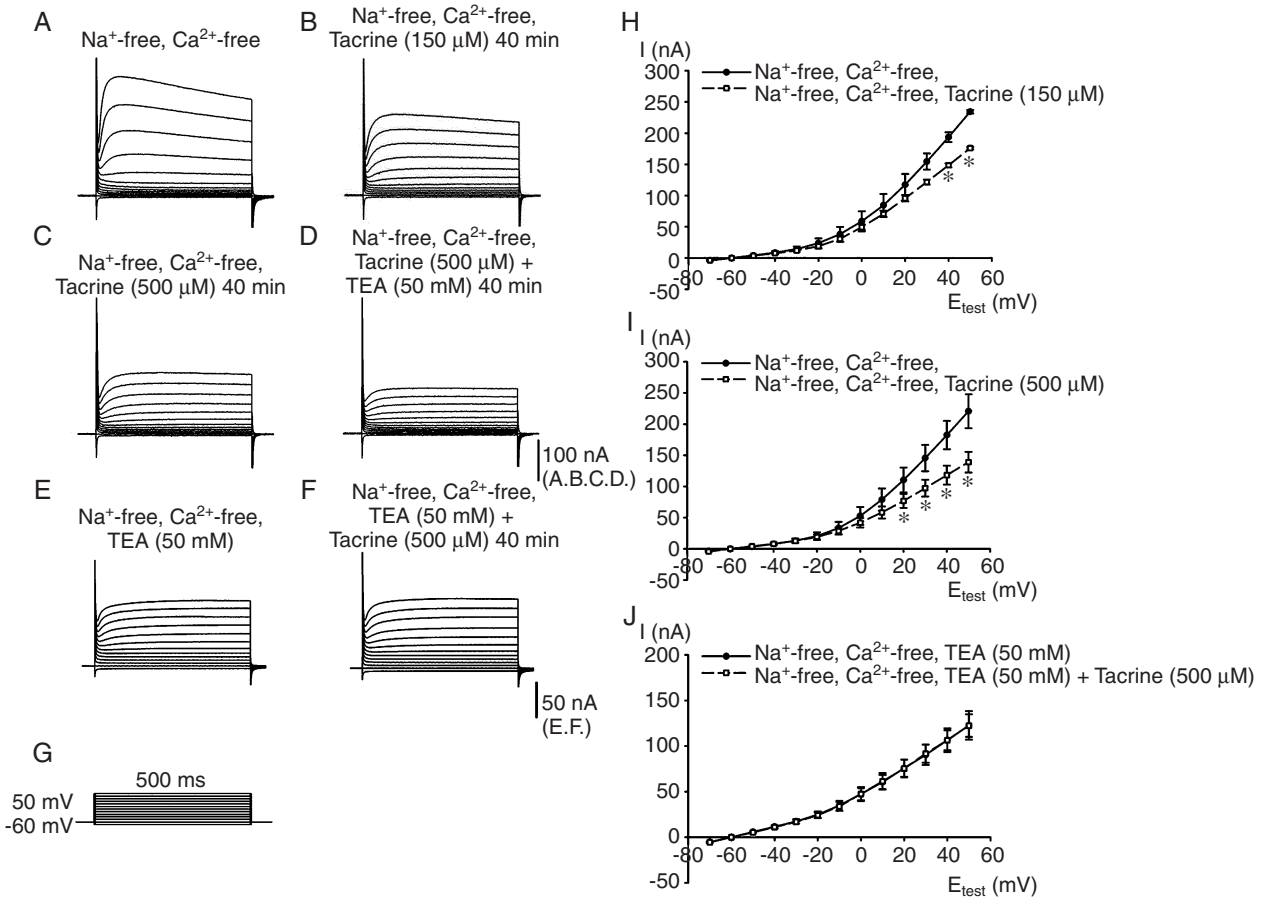

Fig. 12. Effects of tacrine and TEA on steady-state outward currents of RP1 neuron. The Ca^{2+} current and Na^+ current were removed using Co^{2+}-substituted Ca^{2+}- free saline and Na^+-free saline. A, B, C and D were recorded from one RP1 neuron, while E and F were from another. A: Steady-state outward currents recorded in Na^+-free saline and Co^{2+}-substituted Ca^{2+}-free saline. B: The currents at 40 min after tacrine at 150 μM was further incubated from A. C: The currents at 40 min after tacrine at 500 μM administration from B. D: The currents at 40 min after TEA at 50 mM was further incubated from C. E: The currents in TEA -containing Na^+-free saline and Co^{2+}-substituted Ca^{2+}-free saline. F: The currents at 40 min after tacrine at 500 μM was further incubated from E. G: The voltage step commands. H and I: Current-voltage relationships of the steady-state outward current before (●) and after (□) tacrine at 150 μM and 500 μM on the I-V relationships of the steady-state outward currents of the RP1 neuron in Co^{2+}-substituted Ca^{2+}-free and Na^+-free solution, respectively. *: $P < 0.05$ versus Na^+-free, Ca^{2+}-free group, paired *t*-test, n = 3 and n = 4 in H and I, respectively. J: Current-voltage relationships of the steady-state outward current before (●) and after (□) tacrine (500 μM) in TEA-containing Co^{2+}-substituted Ca^{2+}-free and Na^+-free solution, n = 3.

$I_{K(Ca)}$ and I_{KD} are sensitive to TEA (9, 40, 50). For testing the effects of procaine on outward currents, I_{KD}, and I_A were initially measured in the present study.

The measurement of I_A followed Thompson's method (50). We found that at 40 and 60 min after procaine application at 10 mM, the peak I_A current was decreased at test potentials of -30 mV, respectively (Fig. 5), and the I_A current was completely abolished if the bath solution was infused with 4-AP at 5 mM for 40 min. It is noted that perfusion with 4-AP elicited no bursts of action potentials in the RP1 neuron (Fig. 11A). The results suggest that the effect of procaine at 10 mM on the I_A current may not directly be related to its effect on bursts of action potentials.

To measure I_{KD} currents of the RP1 neuron, 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 (23). Under this condition, the $I_{K(Ca)}$, Ca^{2+} and Na^+ currents 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 at 50 mM and remained unaffected by 4-AP at 5 mM (Fig. 6). The sensitivity of the outward current to TEA indicates that this is a type of I_{KD}. Interestingly, a sustained residual current was insensitive to TEA.

We further tested whether procaine at 10 mM affects the steady-state outward current and its time course. We found that perfusion with procaine for 20

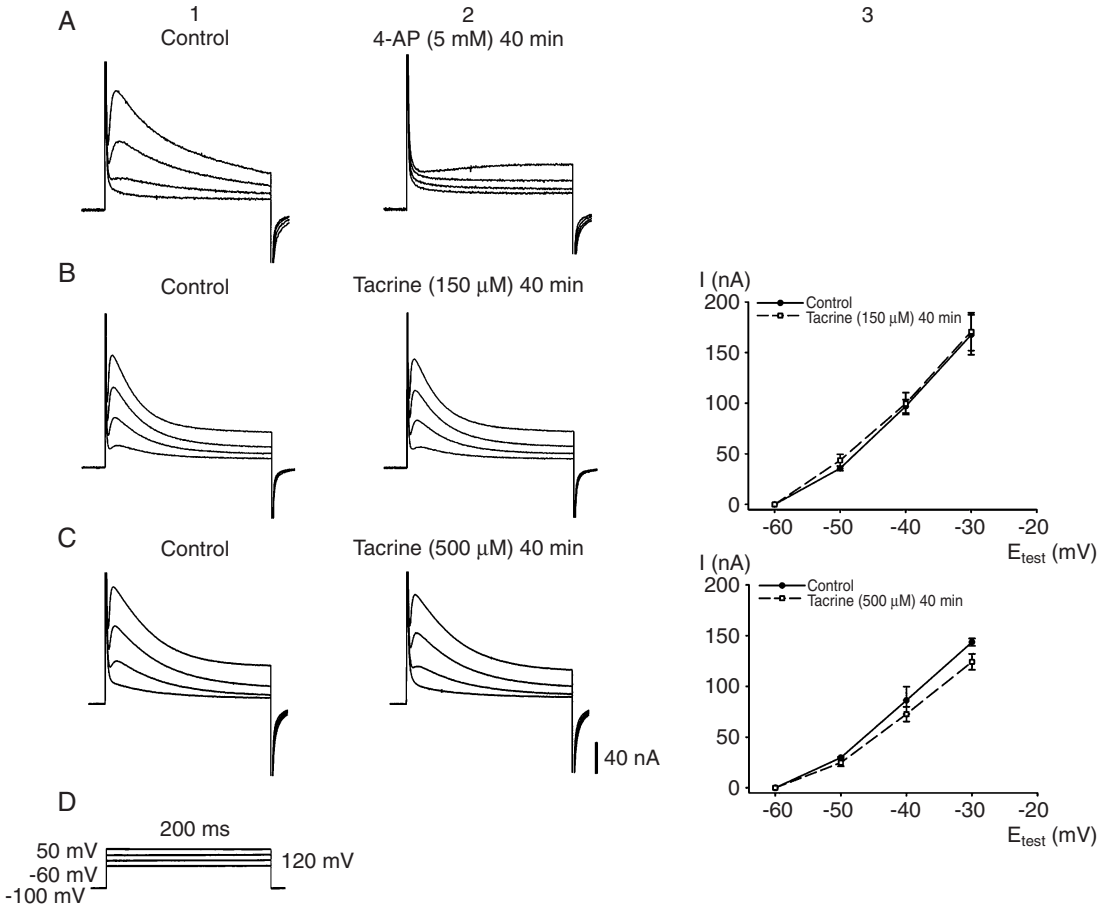

Fig. 13. Effects of tacrine on the peak amplitude of the I_A current of the RP1 neuron. A1, B1 and C1: Control I_A currents recorded in Na^+-free solution. D1: The voltage step commands. A2: I_A currents recorded at 40 min after 4-AP at 5 mM administration from A1. B2 and C2: I_A currents recorded at 40 min after tacrine at 150 μM and tacrine at 500 μM administration from B1 and C1, respectively. B3 and C3: Current-voltage relationships of the I_A currents before (●) and after (□) tacrine (150 μM and 500 μM), respectively.

or 40 min did not significantly decrease the steady state outward current of the RP1 neuron in a Na^+-free and Co^{2+}-substituted Ca^{2+}-free solution. However, continuous perfusion for 60 min did decrease the steady state outward current in Na^+-free and Co^{2+}-substituted Ca^{2+}-free solution (Fig. 7). The current was not further decreased by the addition of TEA at 50 mM for 60 min (Fig. 8G). It seems that application of procaine for 60 min inhibits a TEA-sensitive current in the Na^+-free and Co^{2+}-substituted Ca^{2+}-free solution. It is noted that the time course of inhibitory effects on the steady-state outward current is consistent with that of action potential burst generations, *i.e.* at 60 min after application of procaine.

Tacrine is a centrally acting anticholinesterase agent that reportedly only inhibits I_{KD} without affecting other ionic currents in muscle cells of *Drosophila* (23). In our experiments, tacrine at 150 μM and 500 μM elicited action potential bursts (Fig. 11) and inhibited the steady-state outward current in Na^+-free and Co^{2+}-substituted Ca^{2+}-free solution (Fig. 12). It is noted that tacrine at 500 μM did not further decrease the steady-state outward current when I_{KD} had already been blocked by TEA (Fig. 12J). These results indicate that tacrine decreases the I_{KD} in the RP1 neuron. In our study, we also tested the effects of tacrine on I_A and found that tacrine did not significantly affect the I_A (Fig. 13). The results are consistent with the results of the previous study (23).

U73122 at 10 μM, known as a phospholipase C inhibitor (15), blocked the initiation of action potential bursts elicited by procaine at 10 mM on the central RP1 neuron of the giant African snail (*Achatina fulica* Ferussac) (Fig. 9). In this voltage-clamp study, we found that U73122 did not affect the steady-state outward current in the Na^+-free and Co^{2+}-substituted Ca^{2+}-free solution (Figs. 10A2 and 10B), which suggests that U73122 failed to affect the I_{KD} current.

The phospholipase C (PLC) signaling system

constitutes a virtually universal signal-transduction mechanism in both neural and non-neural cells (7). A recent study reported that extracellular application of the membrane-permeant LAs bupivacaine selectively inhibited G protein-gated inwardly rectifying K^+ channels (GIRK:Kir3) (55). Those authors also suggested that membrane-permeant LAs inhibit GIRK channels by antagonizing the interaction of phosphatidylinositol 4,5-bisphosphate (PIP2) with the channels. In the present study, U73122 at 10 μM decreased the procaine-elicited changes to steady-state outward currents (Fig. 10B). As a PLC inhibitor, U73122 is supposed to decrease the hydrolysis of membrane PIP_2 to yield the diffusible messenger inositol 1,4,5-trisphosphate (IP_3) and the membrane-associated fatty acid diacylglycerol (DAG) in the snail neuron. It is, therefore, assumed that the decrease in the hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) may have decreased the effects of procaine on the I_{KD}. However, the role of PLC and PIP_2 in the inhibitory effects of procaine on the ionic current remains a topic for further studies.

It is reported that LAs act beyond the blocking of Na^+ channels. The voltage-dependent sodium channel is believed to be the primary target for LAs, which also have several other pharmacological actions, including blocking of calcium and potassium channels (22, 55) and inhibition of *N*-methyl-d-aspartate receptors (45). In snail neurons, lidocaine has been reported to induce depolarization in each pre- and postsynaptic neuron by blocking the potassium channel (33). The depolarization of presynaptic neurons may cause lidocaine to inhibit cholinergic synaptic transmission (34).

Procaine has been used as a specific limbic epileptic focus activator in rodents and to induce seizure-like EEG patterns in the absence of seizures (1). However, it remains unclear as to how procaine elicits seizures in animals. In our previous study, we reported that procaine elicited action potential bursts in neurons. In the present study, we found that procaine decreased [1] the Ca^{2+} current, [2] the Na^+ current, [3] the I_{KD} and [4] the I_A currents in the central snail RP1 neuron, yet the inhibitory effect of procaine on the I_{KD} is responsible for the generation of action potential bursts. Further, U73122 decreases the effects of procaine on I_{KD} inhibition and action potential bursts thereby suggesting that these effects are related to PLC activity.

Acknowledgments

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