Doi: 10.1111/j.1742-7843.2007.00118.x
 Basic & Clinical Pharmacology & Toxicology, **101**, 269–276

Bursts of Potential Elicited by *d*-Amphetamine in Central Snail Neuron: Effect of Sodium Azide

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(Received April 25, 2007; Accepted May 14, 2007)

Abstract: Effects of sodium azide (NaN_3) on spontaneously generated action potential and bursts of potential elicited by *d*-amphetamine (*d*-amphetamine-elicited BoP) were studied on the right parietal 4 (RP4) neuron of the snail *Achatina fulica* Ferussac *in vitro*. Sodium azide altered the spontaneous action potential of RP4 neuron in a concentration-dependent manner. In lower concentrations, neither NaN_3 (30, 100, 300 μM; 1 and 3 mM) nor *d*-amphetamine (135 μM) affect the resting membrane potential, amplitude and frequency of RP4 neurons, while in the higher concentrations NaN_3 (30 mM) did abolish the spontaneous action potential on RP4 neurons and depolarized the RP4 neurons reversibly. At lower concentration, NaN_3 (30 μM) facilitated the *d*-amphetamine-elicited BoP. The BoP elicited by NaN_3 (30 μM) and *d*-amphetamine (135 μM) were decreased following treatment with KT5720 (protein kinase A inhibitor), or intracellular injection of EGTA [ethylene glycol-bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid]. However, the BoP was not affected by applying U73122 (1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione) or neomycin (phospholipase inhibitors). Voltage clamp studies revealed that NaN_3 (30 μM) did not alter the total fast inwards currents (70 msec.) and the steady-state outwards currents (5 sec.). It appeared that the BoP elicited by NaN_3 (30 μM) and *d*-amphetamine (135 μM) was mainly due to protein kinase A-related messenger system and intracellular calcium. It is concluded that *d*-amphetamine-elicited BoP was not mainly due to inhibition of the function of mitochondria in the neuron while the function of mitochondria did alter the BoP elicited by amphetamine.

Amphetamine is a compound that enhances not only the locomotor activity of mice, but also the psychic states of human beings [1]. It also releases dopamine in the brain, and amphetamine can exacerbate schizophrenic symptoms or even initiate symptoms of paranoid schizophrenia. These responses are blocked by dopamine antagonists such as chlorpromazine [2]. However, dopamine receptor blockers do not protect against seizures elicited by amphetamine [3].

Our previous studies revealed that *d*- or *l*-amphetamine-elicited bursting firing of potential changes in tight-seal, whole-cell recording in neurons from thin slices of neonatal rat ventrobasal thalamus and in isolated right parietal 1 (RP1) and RP4 neurons of the giant African snail reversibly. The left parietal 4 (LP4), LP5 and LP6 neurons from the same snail, however, were not affected even after higher concentrations of *d*-amphetamine treatment (0.8 mM). It appears that *d*-amphetamine has selective activity on neurons. Neither activation of ganglionic, nicotinic or muscarinic cholinergic receptors, nor activation of the releasing process of the transmitters affected the amphetamine-elicited bursting of potential (BoP), while the amphetamine-elicited BoP was modulated by second messengers [4,5].

The action potential of excitable membrane was modulated by mitochondria inhibitors [6]. Sodium azide (NaN_3) was used mainly as a preservative in aqueous laboratory reagents and biologic fluids, and as a fuel in automobile airbag gas generants [7]. Sodium azide was a cytochrome c oxidase inhibitor [8] and it altered the function of mitochondria. Chronic administration of NaN_3 in rats inhibits cytochrome c oxidase and produces learning and memory deficits and it also significantly decreases membrane-bound protein kinase C activity in hippocampus [9]. Whether NaN_3 can alter the *d*-amphetamine-elicited BoP remains unknown.

To test whether the function of mitochondria was involved in the generation of the bursting firing of action potentials elicited by amphetamine, the effects of NaN_3, cytochrome oxidase inhibitor were evaluated. We found that NaN_3, at lower concentrations, did not alter the electrophysiological properties of the RP4 neuron, while it did facilitate the BoP elicited by *d*-amphetamine. The facilitation effect was related to protein kinase A and intracellular calcium ion. It is concluded that the function of mitochondria do alter the BoP elicited by amphetamine.

Materials and Methods

Experiments were performed on identified central RP4 neurons from the suboesophageal ganglia of the African snail, *Achatina fulica* Ferussac. The ganglia were pinned to the bottom of a 3.0-ml sylgard-coated perfusion chamber and carefully freed from the connective tissue sheath to allow easy identification and penetration by microelectrodes [5,10,11].

For intracellular recording, a Gene clamp 500 amplifier (Axon Instruments Inc., Foster City, CA, USA) was used. Microelectrodes

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(5.0~8.0 MΩ) for recording membrane potentials were filled with 3.0 M KCl. The experimental chamber was perfused with control saline; composition (mM): NaCl, 85.0; KCl, 4.0; $CaCl_2$, 8.0; $MgCl_2$, 7.0; Tris-HCl, 10.0 (pH 7.6) at 23.0~24.0°C [11]. For the quality of neurons tested, neurons were studied only if they had resting membrane potentials (RMP) more negative than –50 mV with the time constant at about 5~8 msec. and the rate of rise of the action potentials at about 5~8 ΔV/sec. Sodium azide and other drugs were applied by extracellular incubation. All potentials were recorded on tape by a digitalizing unit (Digidata 1200; Axon Instruments Inc.) and analysed.

For testing the effects of NaN_3 on RMPs, amplitudes of action potentials and the frequency of single spikes of action potentials of the RP4, RMPs, amplitudes of action potentials and the frequency of single spikes of action potentials were recorded 60 min. after NaN_3 administration.

To test the effects of NaN_3 on *d*-amphetamine-elicited BoP, the RP4 neuron was first treated with NaN_3 for 60 min., and then *d*-amphetamine was further added for 60 min.

To test the effects of KT5720, (9S,10S,12R)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid hexyl ester (protein kinase A inhibitor), on NaN_3 and *d*-amphetamine-elicited BoP, the RP4 neuron was first treated with KT5720 for 60 min., and then NaN_3 and *d*-amphetamine were added [5]. To test the effects of neomycin or U73122 (1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione) on NaN_3 and *d*-amphetamine-elicited BoP, neomycin or U73122 was added for 60 min. after NaN_3 and *d*-amphetamine-elicited BoP.

For intracellular injections of EGTA [ethylene glycol-bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid] into the RP4 neuron, EGTA (250 mM) was dissolved in 100 mM KCl and then the injections were made by pulse under manual control over the course of the experiment [11]. The EGTA in injected neuron was around 5 mM.

For testing the role of NaN_3 on the ionic current, voltage clamp methods were studied. For voltage clamping, the neurons were clamped by means of a Gene clamp 500 amplifier (Axon Instrument Inc.). All potentials and currents were recorded on tape via a digitalizing unit (Digidata 1200) and analysed using a pCLAMP system (Axon Instrument Inc.). For peak amplitude of total inwards current, the currents were elicited by 70 msec. of –50 to +30 mV from a holding potential of –60 mV. For steady-state outwards currents, the currents were elicited by 5 sec. of –100 to +20 mV from a holding potential of –60 mV.

The mean amplitude of the potentials after various treatments was compared to the pre-drug control by means of Student's two-tailed t-test. Statistical data were considered significant differences with $P < 0.05$ [12].

All drug stocks were made with doubly distilled water except for U73122 and KT5720, which were prepared in dimethyl sulfoxide. The presence of dimethyl sulfoxide (≤0.1%) alone did not affect the RMP, amplitude and frequency of the spontaneous firing of action potential in the RP4 neuron.

All procedures used in this study were approved by the Animal Ethics Committee of National Taiwan University in accordance with internationally accepted principles for the care and use of experimental animals. All efforts were made to minimize the number of animals used and their suffering.

Results

The identifiable RP4 neuron of Achatina fulica *Ferussac.*

The central RP4 neuron [13], the identified neuron in the periodically oscillating neuron, from the suboesophageal ganglia of the African snail, *Achatina fulica* Ferussac, is sensitive to several neurotransmitters. Glutamic acid (50 μM) induced hyperpolarization of the membrane potential [5]. Serotonin (5-HT) (50 μM), γ-aminobutyric acid (GABA) (50 μM), dopamine (50 μM) or acetylcholine (ACh) (50 μM) increased the frequency of the spontaneous action potential of the neuron [5].

The RP4 neuron had a resting membrane potential of –56.1 ± 0.9 mV (n = 10, mean ± S.E.M.), and it showed a spontaneous firing of action potential at a frequency of 45.8 ± 2.3 pulses/min. (n = 10). The action potentials show a regularly spaced single spike. No bursts firing of action potential is observed in control RP4 neurons. The mean amplitude of the spontaneously generated action potential was 89.4 ± 1.1 mV (n = 10).

Effects of NaN_3 on the RP4 neuron.

Effects of NaN_3 (30 μM~30 mM) on the RP4 neuron are shown in table 1 and figs 1 and 2. Sodium azide did not alter the RMP, amplitude and frequency of action potential of the RP4 neuron at the lower concentrations tested (30 μM~3 mM), but NaN_3 altered the RMP and amplitude of the spontaneous action potential at the higher concentrations (10 and 30 mM) tested. The changes of potential elicited by NaN_3 were recovered to control level after 120 min. of continuous washing off NaN_3 with normal saline (figs 1 and 2). It appears that higher concentration of NaN_3 (30 mM) reversibly abolished the spontaneous action potential on the RP4 neuron.

Table 1.

Effects of NaN_3 on the resting membrane potential, amplitude and frequency of spontaneously generated action potential of RP4 neurons. The data were recorded after application of NaN_3 (30 μM~10 mM) for 60 min. and NaN_3 (30 mM) for 20 min.

	Variable	RMP (mV)	Amplitudes (mV)	Frequency of single spikes (pulses/min.)
NaN_3	Control (n = 40)	–56.1 ± 0.9	89.4 ± 1.1	45.8 ± 2.3
	30 μM (n = 4)	–55.7 ± 3.0	90.6 ± 1.9	43.1 ± 2.2
	100 μM (n = 6)	–55.3 ± 4.9	87.1 ± 2.9	42.0 ± 10.7
	300 μM (n = 8)	–53.7 ± 2.7	83.5 ± 4.4	42.6 ± 4.3
	1 mM (n = 5)	–53.7 ± 3.8	85.5 ± 5.8	42.0 ± 7.3
	3 mM (n = 6)	–51.0 ± 4.2	86.2 ± 1.6	40.5 ± 7.8
	10 mM (n = 8)	–47.0 ± 3.1*	71.5 ± 4.8*	48.7 ± 4.1
	30 mM 20 min. (n = 5)	–38.9 ± 1.9*	–	–

RMP, resting membrane potential.
Values were expressed as the mean ± S.E.M. (n is the number of neurons tested).
*Statistically significant compared with the data in physiological solution (control), $P < 0.05$.

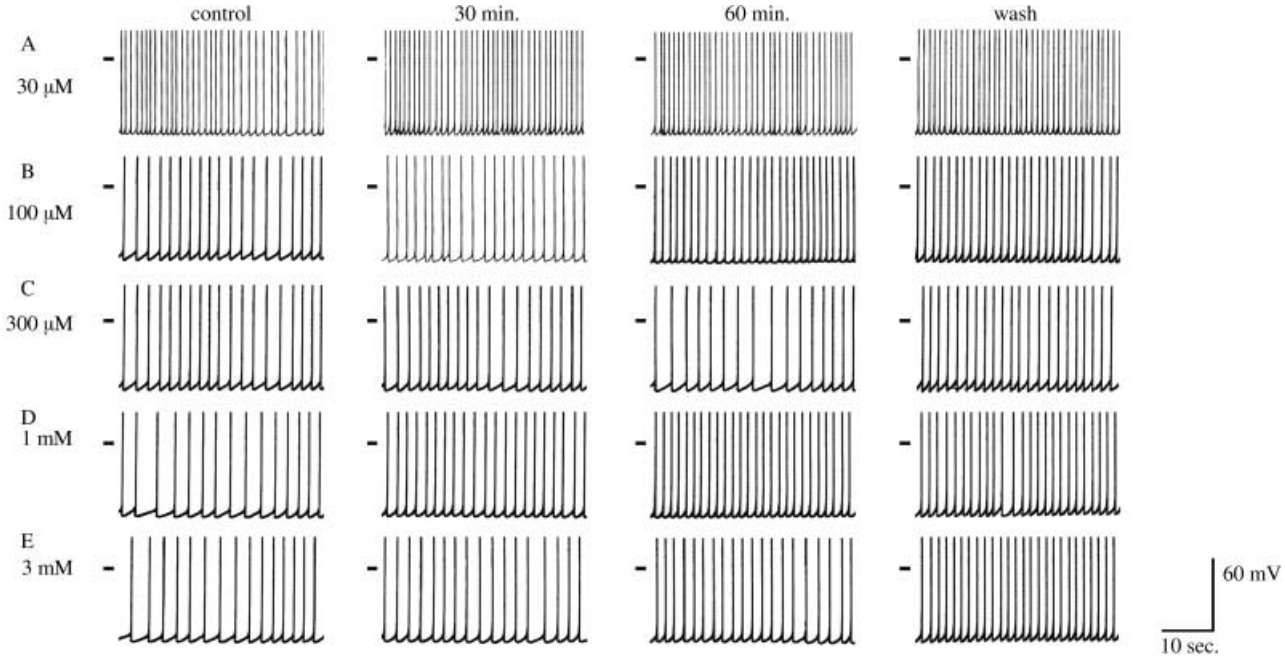

Fig. 1. Effects of NaN_3 on the central RP4 neuron of snails. Control in A, B, C, D and E were potentials of control in the right parietal 4 (RP4) neurons. At 30 and 60 min. in A, B, C, D and E were potentials after 30 and 60 min. of application of NaN_3 (30, 100, 300 μM; 1, and 3 mM), respectively. At wash in A, B, C, D and E were potentials after 120 min. of washing off with normal saline from preparations incubated with 60 min. NaN_3 of A–E, respectively. The horizontal bar at the top left indicates the membrane potential at 0 mV.

Effects of NaN_3 on the d-*amphetamine-elicited bursting of action potentials.*

Sodium azide (30 μM) does not affect the resting membrane potentials and the frequency of the spontaneously generated action potentials of the neuron. The resting membrane potential of the identified RP4 neuron was –59.4 ± 3.3 mV (n = 4, mean ± S.E.M.). One hundred twenty minutes after application of NaN_3 (30 μM), the resting membrane potential was –58.5 ± 1.5 mV (n = 4, P > 0.05). The spontaneous frequency of potentials in control and incubation with *d*-amphetamine (135 μM) for 60 min. were 39.8 ± 5.3 pulses/min. (n = 5) and 36.0 ± 4.4 pulses/min. (n = 5, P > 0.05), respectively. No bursting firing of action potentials was observed in *d*-amphetamine (135 μM)-treated preparations

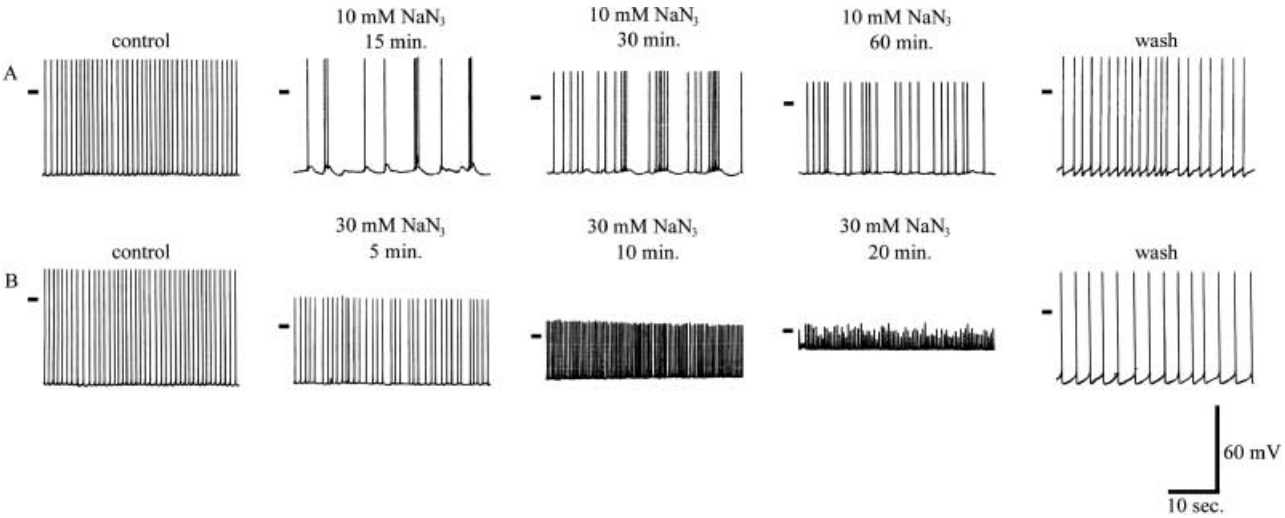

Fig. 2. Effects of NaN_3 on the central RP4 neuron of snails. Control in A and B were potentials of control in the right parietal 4 (RP4) neurons. At 15, 30 and 60 min. in A were potentials after 15, 30 and 60 min. of application of NaN_3 (10 mM), respectively. At 5, 10 and 20 min. in B were potentials after 5, 10 and 20 min. of application of NaN_3 (10 mM), respectively. At wash in A and B were potentials after 120 min. of washing off with normal saline from preparations after treatment from A, and B, respectively. The horizontal bar at the top left indicates the membrane potential at 0 mV.

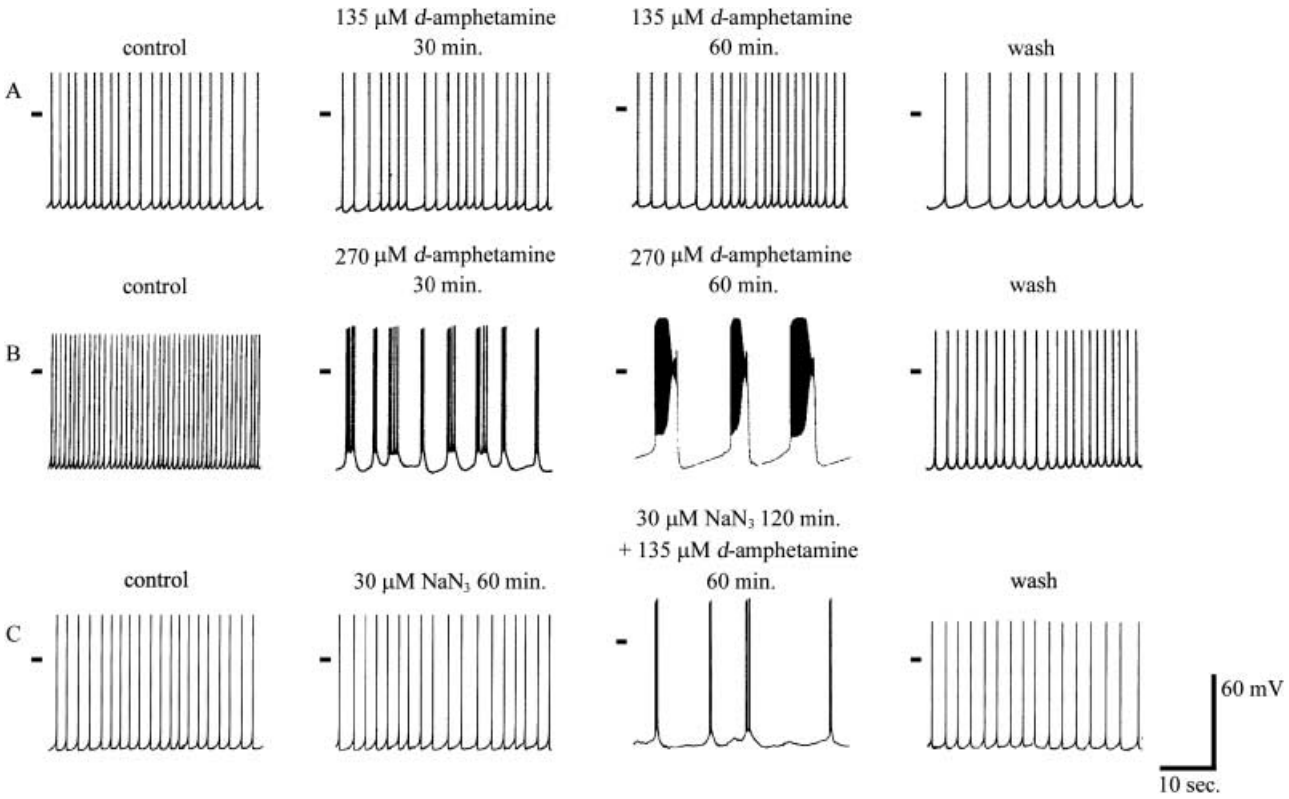

Fig. 3. Sodium azide facilitated the *d*-amphetamine-elicited BoP on right parietal 4 (RP4) neurons. Control in A, B and C were potentials of control in the RP4 neurons. At 30 and 60 min. in A and B were potentials after 30 and 60 min. of application of *d*-amphetamine (135 and 270 μM), respectively. C were potentials after 60 min. of application of NaN_3 (30 μM) or after 60 min. after application of *d*-amphetamine (135 μM) and 120 min. of NaN_3 (30 μM). At wash in A, B and C were potentials after 120 min. of washing off with normal saline from preparations after treatment from A, B and C, respectively. The horizontal bar at the top left indicates the membrane potential at 0 mV.

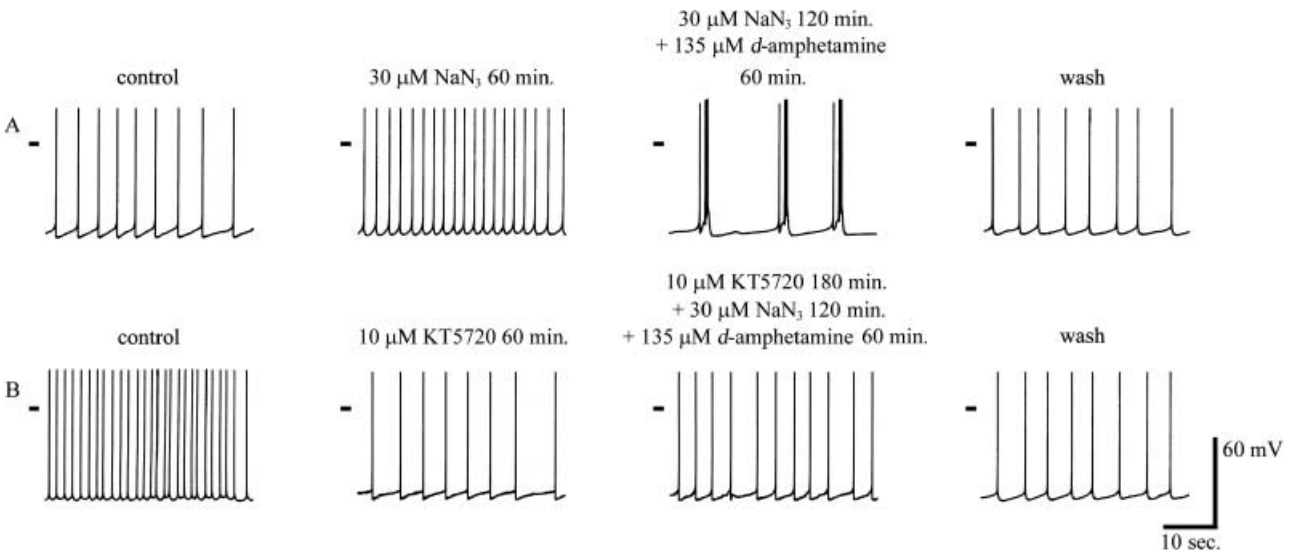

Fig. 4. Effect of KT5720 (protein kinase A inhibitor) on the BoP elicited by NaN_3 and *d*-amphetamine on right parietal 4 (RP4) neurons. Control in A and B were potentials of control in the RP4 neurons. A showed NaN_3 (30 μM) reversibly facilitated the *d*-amphetamine (135 μM)-elicited bursting of potential (BoP). B showed KT5720 reversibly blocked the BoP facilitated by NaN_3 (30 μM) and *d*-amphetamine (135 μM). At wash in A and B were potentials after 120 min. of washing off with normal saline from preparations after treatment from A and B, respectively. The horizontal bar at the top left indicates the membrane potential at 0 mV.

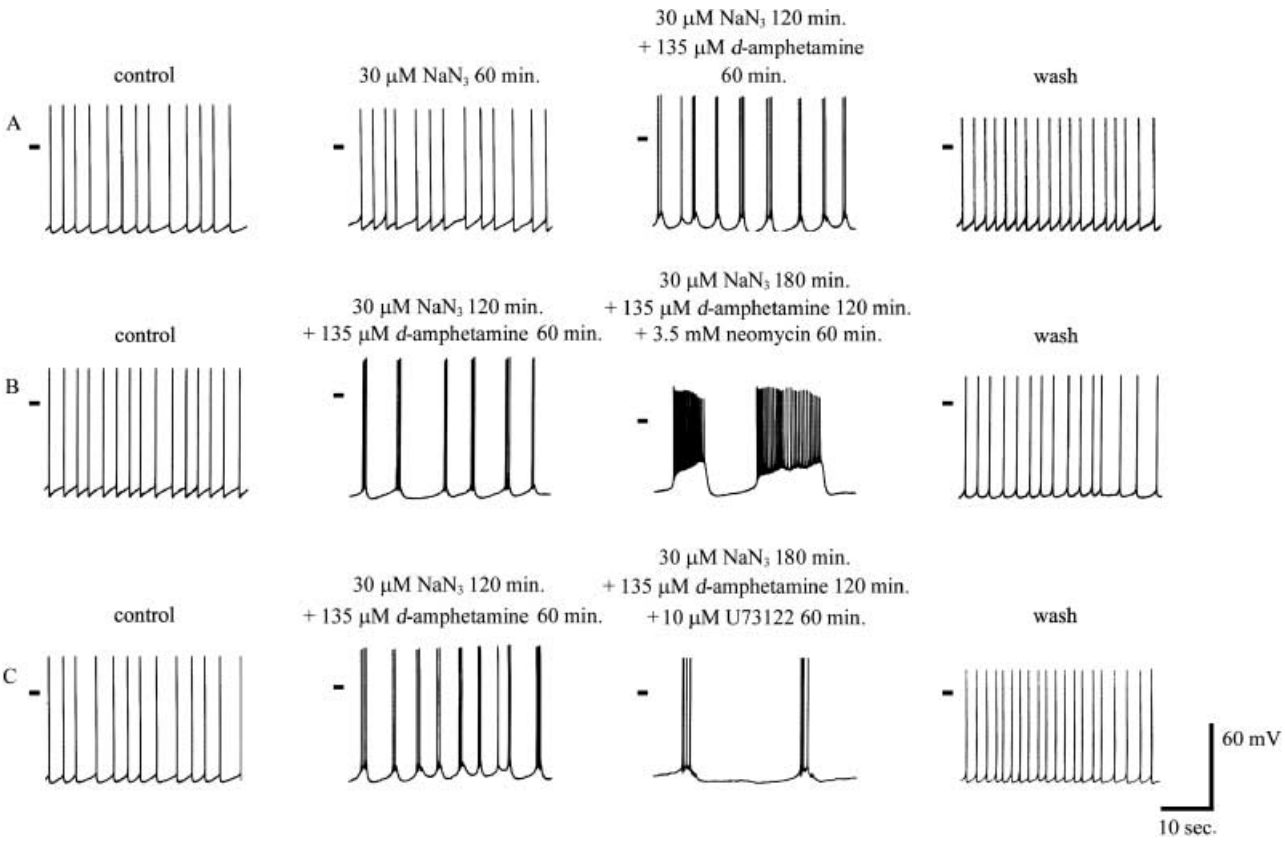

Fig. 5. Effect of neomycin and U73122 (1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione) on the bursts of potentials elicited by NaN_3 and *d*-amphetamine on right parietal 4 (RP4) neurons. Control in A, B and C were potentials of control in the RP4 neurons. A showed NaN_3 (30 μM) reversibly facilitated *d*-amphetamine (135 μM)-elicited bursting of potential (BoP). B or C showed BoP facilitated by NaN_3 (30 μM) and *d*-amphetamine (135 μM) were not altered in the presence of neomycin or U73122. At wash in A, B and C were potentials after 120 min. of washing off with normal saline from preparations after treatment from A, B, and C, respectively. The horizontal bar at the top left indicates the membrane potential at 0 mV.

(fig. 3). It seems that *d*-amphetamine (135 μM) alone does not elicit the bursting of action potentials in the RP4 neuron.

The effects of NaN_3 (30 μM) on the bursting of potentials elicited by *d*-amphetamine were tested in RP4 neurons. *d*-Amphetamine at 135 μM did not elicit bursting of potentials in the RP4 neuron after 60-min. incubation. However, if NaN_3 (30 μM) was applied to the preparation before adding lower concentrations of *d*-amphetamine (135 μM), bursting firing of action potentials were found 60 min. after application of *d*-amphetamine (135 μM). The BoP was recovered to control level if both NaN_3 and amphetamine were washed out (fig. 3). It appears that NaN_3 (30 μM) reversibly facilitated the generation of BoP elicited by *d*-amphetamine (135 μM).

Effects of KT5720, neomycin or U73122 on the potentials elicited by NaN_3 and d-*amphetamine.*

To test whether the 3′-5′-cyclic adenosine monophosphate (cAMP)-dependent protein kinase system or phospholipase C is involved in the generation of the BoP elicited by NaN_3 and *d*-amphetamine, the effects of KT5720, neomycin or U73122 are evaluated.

After perfusion of KT5720 (10 μM) for 60 min., the resting membrane potential, amplitude and frequency was –57.5 ± 0.5 mV (n = 3, P > 0.05), 94.3 ± 2.3 mV (n = 3, P > 0.05) and 27.0 ± 9.0 pulses/min. (n = 3, P < 0.05). The resting membrane potential and amplitude of the RP4 neuron were not affected by KT5720 (10 μM) (P > 0.05), but the frequency was decreased significantly (P < 0.05). Even perfusion of KT5720 (10 μM) for 180 min. did not evoke the BoP on the RP4 neuron. KT5720 (10 μM) was applied 60 min. before NaN_3 (30 μM) and *d*-amphetamine (135 μM) elicited the BoP on RP4 neuron. The BoP elicited by NaN_3 (30 μM) and *d*-amphetamine (135 μM) disappeared, and the resting membrane potential, amplitude and frequency of RP4 neuron was –52.0 ± 4.0 mV (n = 3), 93.5 ± 2.5 mV (n = 3) and 36.0 ± 6.0 pulses/min. (n = 3). An example of the effects of KT5720 on the potentials elicited by NaN_3 and *d*-amphetamine is shown in fig. 4.

After BoP elicited by NaN_3 (30 μM) and *d*-amphetamine (135 μM) on the RP4 neuron, the BoP still existed after neomycin (3.5 mM) or U73122 (10 μM) treated for 60 min., as shown in fig. 5. Similar results were found in three other preparations. It appears that BoP elicited by NaN_3 (30 μM) and *d*-amphetamine (135 μM) is associated

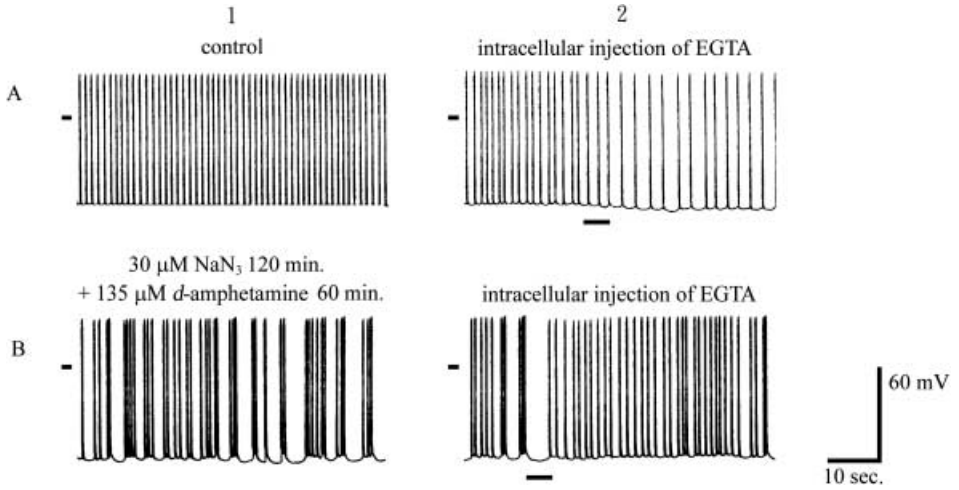

Fig. 6. Effect of intracellular injection of EGTA [ethylene glycol-bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid] on bursts of potential elicited by NaN_3 and *d*-amphetamine on right parietal 4 (RP4) neurons. A1 was the potentials of control. B1 was the potentials after application of 120 min. of NaN_3 (30 μM) and 60 min. of *d*-amphetamine (135 μM). The cross bar at A2 and B2, EGTA (5 mM), was intracellular injected for 5 sec. The horizontal bar at the top left indicated the membrane potential at 0 mV.

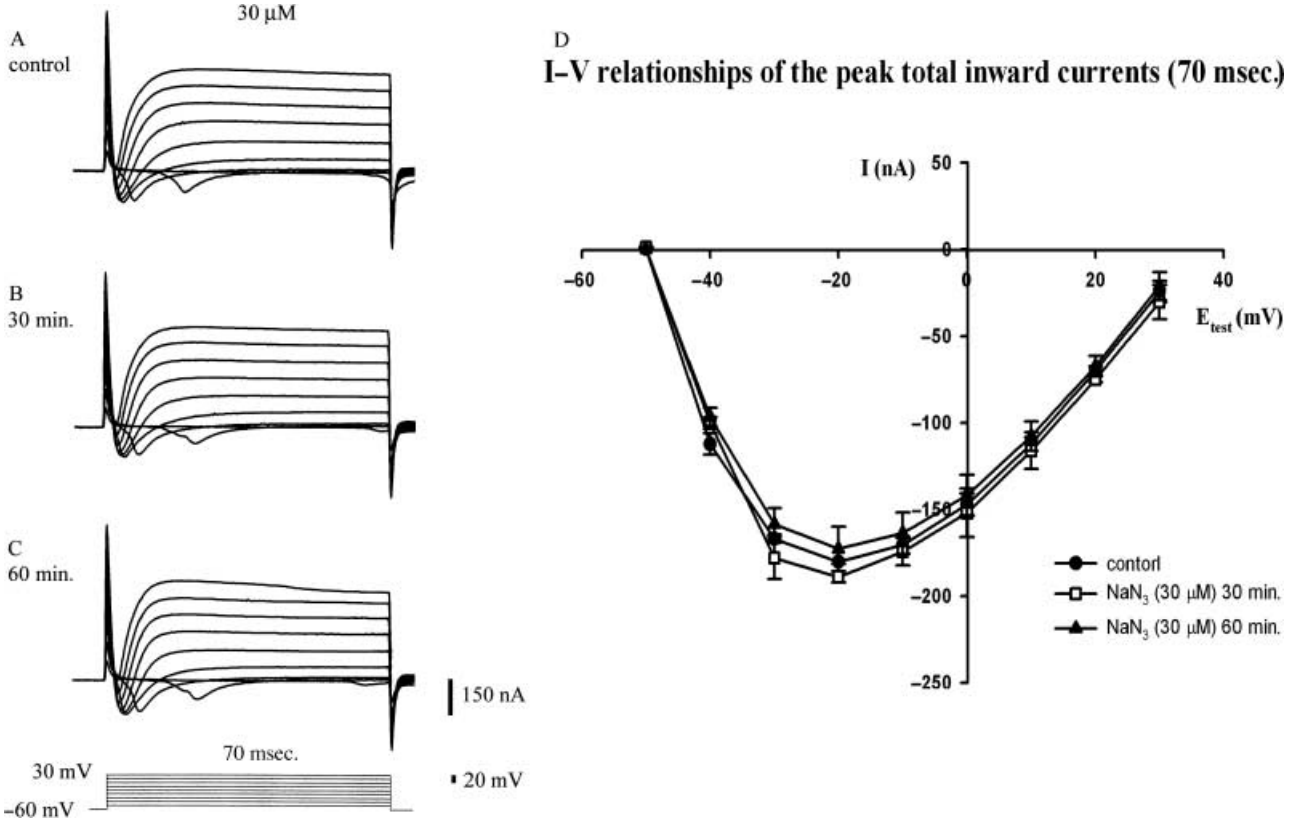

Fig. 7. Effects of NaN_3 (30 μM) on total inwards currents of the right parietal 4 (RP4) neurons. Ion currents were recorded during voltage steps in 10 mV increments between –50 and +30 mV from a holding potential of –60 mV (70 msec.). (A) Control, total inwards currents recorded in normal physiological saline. (B) Total inwards currents recorded 30 min. after incubation of NaN_3 (30 μM) from A. (C) total inwards currents recorded 60 min. after incubation of NaN_3 (30 μM) from A. The lowest trace showed the voltage step commands. (D) Current–voltage (I–V) relationships of the steady-state inwards currents before (●), and after NaN_3 (30 μM) application of 30 min. (□) and 60 min. (▲), respectively.

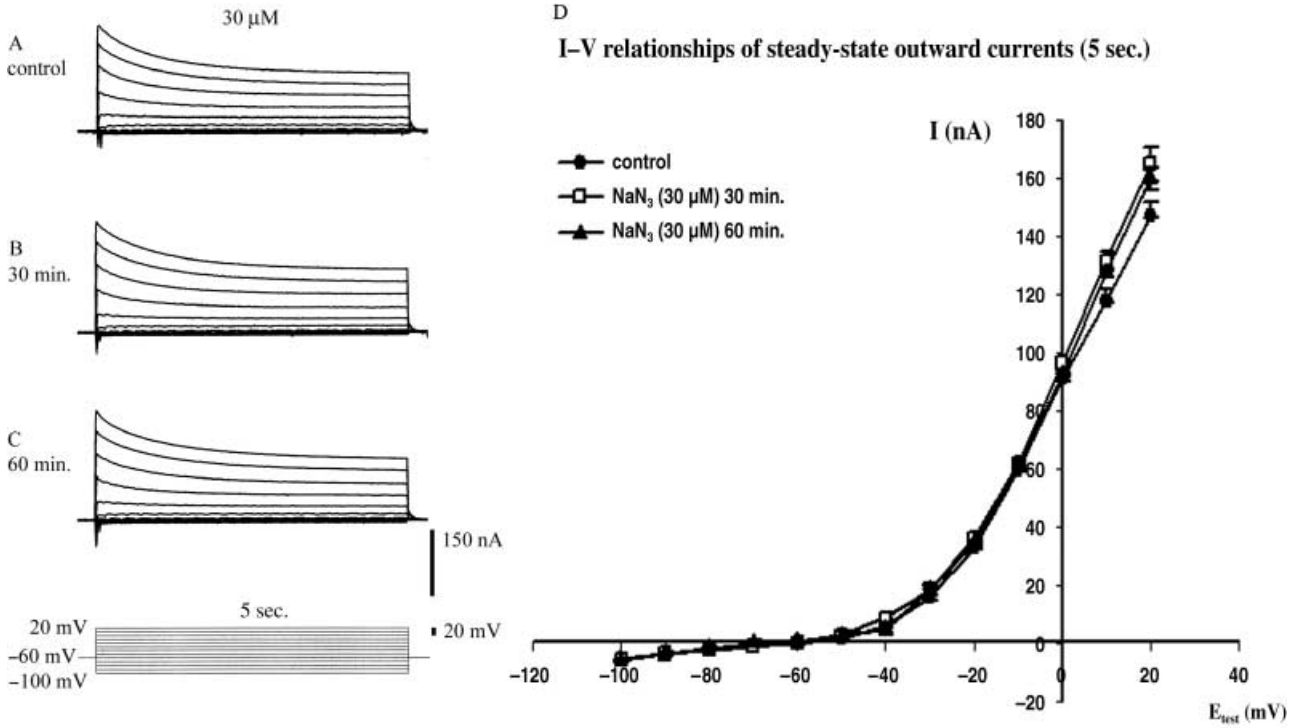

Fig. 8. Effects of NaN_3 (30 μM) on total outwards currents of the right parietal 4 (RP4) neurons. Ion currents were recorded during voltage steps in 10 mV increments between –100 and +20 mV from a holding potential of –60 mV (5 sec.). (A) Control, total outwards currents recorded in normal physiological saline. (B) Total outwards currents recorded 30 min. after incubation of NaN_3 (30 μM) from A. (C) Total outwards currents recorded 60 min. after incubation of NaN_3 (30 μM) from A. The lowest trace showed the voltage step commands. (D) Current-voltage (I–V) relationships of the steady-state outwards currents before (●), after NaN_3 (30 μM) application of 30 min. (□) and 60 min. (▲), respectively.

with protein kinase A, while phospholipase C is not involved in it.

Effects of intracellular calcium on the potential elicited by NaN_3 and d-*amphetamine.*

To test whether calcium ion is involved in the generation of the BoP elicited by NaN_3 and *d*-amphetamine, the EGTA is evaluated.

Upon intracellular injection of EGTA into the RP4 neuron, the resting membrane potential, amplitude and frequency of RP4 neuron were not affected. When BoP elicited by NaN_3 (30 μM) and *d*-amphetamine (135 μM), EGTA was injected intracellularly into the RP4 neuron and the BoP elicited by NaN_3 and *d*-amphetamine was decreased as shown in fig. 6B. It appears that decreasing intracellular calcium ion also decreases the generation of BoP elicited by NaN_3 and *d*-amphetamine.

Effects of NaN_3 on the ionic currents of RP4 neurons.

The effects of NaN_3 (30 μM) on the fast inwards currents of the RP4 neuron during shorter steps (70 msec.) of voltage clamping are shown in fig. 7. The effects of NaN_3 (30 μM) on the current–voltage (I–V) relationship of total inwards currents at various command voltages are shown in fig. 7D. Sodium azide (30 μM) did not significantly decrease the fast inwards currents in a series of voltage steps from –50 to 30 mV ($n = 3$, $P > 0.05$).

Five-second-long stepping pulses are used to measure the steady-state currents of the RP4 neuron. Currents are obtained by stepping from a holding potential of –60 mV to stepping potentials of –100 to 20 mV at intervals of 10 mV in application of NaN_3 (30 μM) as shown in fig. 8. The steady-state currents are measured at 5 sec. after voltage stepping and the steady-state I–V relationships are shown in fig. 8D. Steady-state outwards currents were observed if the positivity of the holding potential was greater than –50 mV. The total outwards current was increased if the command voltage was stepped to a higher positive potential. Sodium azide (30 μM) did not significantly alter the steady-state currents in a series of voltage steps ($n = 3$, $P > 0.05$).

It appears that NaN_3 at 30 μM, the concentration that facilitated BoP elicited by *d*-amphetamine, does not alter the fast inwards or steady outwards ionic currents on the same neuron.

Discussion

Our previous study demonstrated that *d*-amphetamine-elicited bursting firing of potential changes in isolated RP4 neurons of the giant African snail. We also found that

activation of adenylyl cyclase, inhibition of phosphodiesterase, increasing cAMP concentration in the neuron, etc. facilitated the generation of the BoP elicited by *d*-amphetamine [4,5,10,11,13,14]. However, the effect of metabolic inhibitor on the *d*-amphetamine-elicited BoP remains unclear.

In the present study, we found that NaN_3 (30 μM) facilitates the BoP elicited by *d*-amphetamine (135 μM). The BoP elicited by NaN_3 (30 μM) and *d*-amphetamine (135 μM) were decreased following treatment with KT5720 (protein kinase A inhibitor) or intracellular injection of EGTA. However, they were not affected by applying U73122 or neomycin (phospholipase inhibitors). U73122 or neomycin blocked the BoP elicited by procaine in the same spontaneous oscillation neuron [15].

The action potential of excitable membrane is modulated by many factors including mitochondria inhibitors [6]. Sodium azide was a cytochrome c oxidase inhibitor [8]. Chronic administration of NaN_3 inhibited cytochrome oxidase and produced learning and memory deficits, and it also significantly decreased membrane-bound protein kinase C activity in hippocampus in rats [9]. Mitochondria regulated intracellular Ca^{2+} and membrane phospholipids signaling. Sodium azide induced an increase of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) in pancreatic β-cells, as measured by microfluorimetry with digital imaging [16].

Our present result supports the view that NaN_3 facilitated *d*-amphetamine-elicited BoP and the metabolizing inhibitor did alter the potential changes modulated by amphetamine. It is concluded that BoP elicited by amphetamine was not due mainly to the function of mitochondria; however, the BoP elicited by amphetamine was facilitated by the metabolic inhibitor.

Acknowledgements

This work was supported by grant from the National Science Council, NSC-95-2320-B-002–104, Taipei, Taiwan.

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