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Research Report

Paraoxon suppresses Ca^{2+} spike and afterhyperpolarization in snail neurons: Relevance to the hyperexcitability induction

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ABSTRACT

The effects of organophosphate (OP) paraoxon, active metabolite of parathion, were studied on the Ca^{2+} and Ba^{2+} spikes and on the excitability of the neuronal soma membranes of land snail (*Caucasotachea atrolabiata*). Paraoxon (0.3 μM) reversibly decreased the duration and amplitude of Ca^{2+} and Ba^{2+} spikes. It also reduced the duration and the amplitude of the afterhyperpolarization (AHP) that follows spikes, leading to a significant increase in the frequency of Ca^{2+} spikes. Pretreatment with atropine and hexamethonium, selective blockers of muscarinic and nicotinic receptors, respectively, did not prevent the effects of paraoxon on Ca^{2+} spikes. Intracellular injection of the calcium chelator BAPTA dramatically decreased the duration and amplitude of AHP and increased the duration and frequency of Ca^{2+} spikes. In the presence of BAPTA, paraoxon decreased the duration of the Ca^{2+} spikes without affecting their frequency. Apamin, a neurotoxin from bee venom, known to selectively block small conductance of calcium-activated potassium channels (SK), significantly decreased the duration and amplitude of the AHP, an effect that was associated with an increase in spike frequency. In the presence of apamin, bath application of paraoxon reduced the duration of Ca^{2+} spike and AHP and increased the firing frequency of nerve cells. In summary, these data suggest that exposure to submicromolar concentration of paraoxon may directly affect membrane excitability. Suppression of Ca^{2+} entry during the action potential would down regulate Ca^{2+}-activated K^+ channels leading to a reduction of the AHP and an increase in cell firing.

1. Introduction

A key mechanism of action of OPs is the irreversible inhibition of acetylcholinesterase (AChE) and accumulation of acetylcholine (ACh) in cholinergic synapses. This common mechanism explains the similar effects of many OPs and their response to therapy with atropine and oximes (Mileson et al., 1998; Sungur and Guven, 2001). However, cholinergic hyperexcitability cannot account for all of the neurological and behavioral manifestations resulting from acute exposure to OPs. Although the toxic principle of paraoxon has been already described, the mechanism of its action at the cellular

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level remains to be established, and the role of the direct blocking action of OPs on calcium channels is still an open question.

At the cellular level, plasma membrane proteins including ion channels, receptors, and enzymes are the main targets of certain insecticides (Narahashi, 1992; Raymond-Delpech et al., 2005). In particular, paraoxon is known to block $GABA_A$, glycine, NMDA, and ACh receptor channels (Rocha et al., 1996). Some OPs, including parathion and paraoxon, can interact directly with nicotinic and muscarinic receptors (Bakry et al., 1988; Katz and Marquis, 1989; Katz et al., 1997; Silveira et al., 1990; Ward and Mundy, 1996). Paraoxon also blocks chloride channels in *Aplysia* neurons (Filbert et al., 1992). Furthermore, soman, an organophosphorus nerve agent, has been shown to decrease calcium entry through voltage-dependent calcium channels (VDCCs) in bullfrog peripheral sympathetic neurons (Heppner and Fiekers, 1991b).

Calcium homeostasis is a highly controlled process, essential for different intracellular functions including the cell excitability, transmitter release, gene expression, and cell death (Miller, 2001). In neurons, membrane depolarization activates VDCCs leading to large calcium fluxes inside the cell. VDCCs can be considered major structural components that couple neuronal electrical activity of neurons to intracellular calcium signaling.

In the present experiments, conventional intracellular recordings, in current clamp mode, have been used to study the effects of paraoxon on calcium spikes and neuronal excitability in snail neurons, known to bear calcium and sodium channels (Chamberlain and Kerkut, 1969). Our results show that paraoxon at a concentration as low as 0.3 μM produces a reduction in amplitude and duration of Ca^{2+} spike and AHP leading to an increase in the cell excitability, which seems mechanistically unrelated to the cholinesterase inhibition.

2. Results

To determine the non cholinergic effects of paraoxon on the properties of Ca^{2+} spike and neural excitability, paraoxon was applied in a wide range of concentrations from 0.3 up to 2.4 μM. We found that already at 0.3 μM concentration, paraoxon was effective in decreasing the duration of Ca^{2+} spikes and increasing the firing rate. Therefore, in order to minimize the possible anti AChE effects of this drug, we focused on the minimum effective concentration (0.3 μM). In addition, a set of experiments were performed in the presence of ACh receptor antagonists. To characterize the Ca^{2+} spikes in soma membrane of snail neurons, recording was performed after blocking transient outward K^+ (4-AP was added to the bathing solution) and voltage-activated Na^+ inward currents (substitution of TEA for Na^+). Under these conditions, Ca^{2+} spikes showed large amplitude with a plateau phase compared to the fast Na^+ action potential in normal Ringer. In the absence of 4-AP in the bathing solution, as performed in some experiments, neurons did not show the plateau form augmented Ca^{2+} spikes, although the spikes had clearly longer durations (up to hundred milliseconds) in comparison to sodium spikes (a few milliseconds). Experiments were done on 93 neurons, either in Ca^{2+} or Ba^{2+} Ringer. Out of 53 neurons recorded in normal Ca^{2+} Ringer (in the absence of cholinergic receptor antagonists), 35 exhibited spontaneous activity and 18 were silent. Spontaneous Ca^{2+} spikes had a mean amplitude of 78 ± 1.72 mV, duration of 0.31 ± 0.06 s, and a mean firing frequency of 0.13 ± 0.015 Hz (n = 35). Ca^{2+} spikes were followed by a prominent slow decaying AHP with an amplitude of –6.9 ± 0.87 mV and a duration of 4.68 ± 0.19 s (n = 26).

2.1. *Effects of paraoxon (0.3 μM) on intrinsic membrane properties*

Superfusion of ganglia with paraoxon-containing Ca^{2+} Ringer solution produced in 22 out of 27 neurons (15 discharging and 7 silent) a slow and progressive depolarization (within 10 min of paraoxon application from –41.18 ± 0.95 mV to –36.85 ± 0.74 mV; $P < 0.05$). The RMP was slightly shifted toward more negative potential by washout but not as enough to return it to control value, so that it was still significantly depolarized compare to control conditions. In the remaining 5 neurons (3 discharging and 2 silent), RMP remained stable. This effect was associated with a decrease in membrane input resistance (R_{in}) (from 8.21 ± 1.05 MΩ to 7.69 ± 1.28 MΩ; n = 10). The effect of paraoxon on R_{in} was observed in both depolarized neurons and those with stable RMP but was not significant.

2.2. *Effects of paraoxon on Ca^{2+} and Ba^{2+} spikes*

Paraoxon (0.3 μM, applied for 10 min) progressively reduced the amplitude (by 17.8 ± 5.1%; $P < 0.05$) and the duration (by 34.7 ± 7.2%; $P < 0.01$; Figs. 1A, B) of spontaneous Ca^{2+} spikes. The duration and amplitude of the spikes were partially restored after 10 min of washout with a paraoxon free Ca^{2+} Ringer (Fig. 1).

In silent and spontaneously active neurons, depolarizing current pulses (2 nA, 500 ms) evoked action potentials which were followed by a prominent AHP. Application of paraoxon (0.3 μM) significantly reduced the duration of Ca^{2+} spikes from a control value of 0.34 ± 0.08 s to 0.21 ± 0.05 s ($P < 0.01$). The decrease in the duration was accompanied by an increase in the number of spikes per pulse (Fig. 2 left panel). Full recovery was observed after washing out the drug (Fig. 2 left panel). Application of hyperpolarizing pulses was usually followed by rebound Ca^{2+} spikes. Paraoxon also decreased in a reversible manner the duration of rebound spikes which occurred after 10 min of exposure (Fig. 2 right panel). Both in depolarized and nondepolarized neurons, the latency of post-pulse rebound action potentials decreased by paraoxon, but this reduction was more pronounced in depolarized neurons. This effect was not reversible by washout (Fig. 2 right panel).

In most neurons, detectable AHPs were recorded following single Ca^{2+} spikes. When Ca^{2+} spikes were evoked repetitively with depolarizing currents, a summation in duration and amplitude of AHPs was observed (data not shown), suggesting that calcium-dependent potassium channels, possibly apamin sensitive K_{Ca} channels, were involved in AHP generation. In the presence of paraoxon, initially the amplitude and duration of AHP decreased (Fig. 3 middle and right panels). The maximum reduction of duration and amplitude of AHP was recorded within 5 min of paraoxon exposure (33.7 ± 5% and

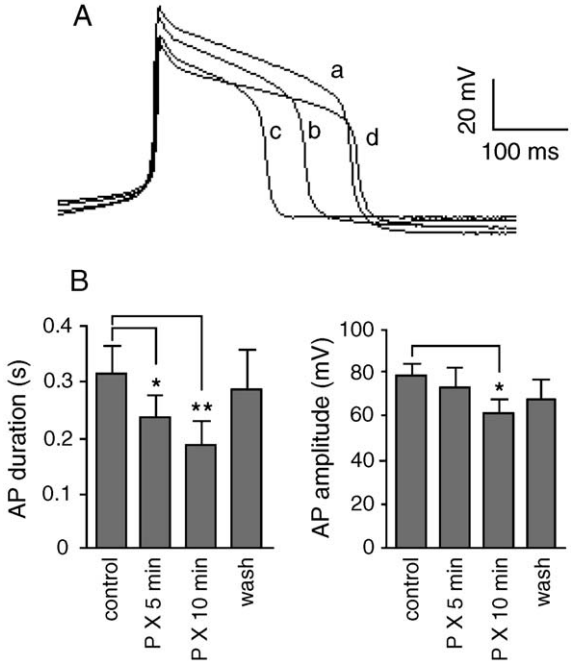

Fig. 1 – Paraoxon reversibly decreased duration and amplitude of spontaneously recorded calcium spike. (A) Ca^{2+} spike traces recorded from a neuron in control (a), 5 min (b), and 10 min (c) after application of paraoxon and after washout (d). (B) Effects of paraoxon (PX) on mean amplitude and duration of Ca^{2+} spikes. (n = 13) $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

28 ± 6.6%, respectively). However, an increase in AHP parameters particularly in the amplitude was observed later (Fig. 3 middle and right panels).

The paraoxon-induced reduction of the AHP was associated with an increase in cell excitability as suggested by the increase in the firing rate (from 0.143 ± 0.017 Hz to 0.218 ± 0.049 Hz; $P < 0.01$). This was followed within 10–15 min by neuronal silencing (n = 17) (Fig. 3 left panel). The paraoxon-induced hyperexcitability occurred also in those cells that were not depolarized by the drug (n = 3) or RMP was maintained at control value by current injection (n = 3).

Ba^{2+} (10 mM) was used as a charge carrier to avoid activation of Ca^{2+}-dependent channel currents. In Ringer containing Ba^{2+}, neurons had a RMP of –38.7 ± 1.8 mV and exhibited spontaneous and evoked Ba^{2+} spikes. They had an amplitude of 80.4 ± 3.3 mV and a duration of 8.19 ± 2.5 s. As expected, they did not show a detectable AHP (n = 12) (Fig. 4). Application of paraoxon within 10 min reduced the duration of Ba^{2+} spikes by 59.8 ± 7.9% ($P < 0.001$; n = 12) (Figs. 4A, B). Spontaneously active neurons had a spike frequency of 0.067 ± 0.018 Hz that was increased to 0.072 ± 0.015 in the presence of paraoxon, but this effect was not significant (n = 6).

2.3. *The effects of paraoxon on Ca^{2+} and Ba^{2+} spikes are independent of cholinergic receptors activation*

To see whether paraoxon affected calcium channels directly (as suggested by the experiments with Ca^{2+} and Ba^{2+} spikes) or indirectly through the activation of cholinergic receptors, additional experiments were performed in the presence of muscarinic and nicotinic receptor antagonists.

In 28 experiments, the ganglia were pretreated for at least 30 min with the specific muscarinic and nicotinic antagonists, atropine (5 μM) and hexamethonium (50 μM). Paraoxon application in the presence of antagonists still was able to modify the Ca^{2+} or Ba^{2+} spike. In the absence of a significant change in R_{in} and RMP, paraoxon (0.3 μM) decreased the Ca^{2+} spike duration by 36.3 ± 4.8% ($P < 0.01$, n = 18) (data not shown). It also significantly ($P < 0.001$) decreased the duration of Ba^{2+} spike by 55.6 ± 7.3% (Fig. 4C) (n = 10) and reduced the amplitude of both Ca^{2+} and Ba^{2+} spikes by 16.5 ± 3.8% and 11.3 ± 4%, respectively. In a calcium- but not barium-containing solution, the paraoxon-induced increase in spike frequency was significant ($P < 0.05$).

2.4. *Effect of paraoxon on the configuration of Ca^{2+} spike after buffering the intracellular Ca^{2+} with BAPTA*

To test whether paraoxon-induced reduction in calcium entry through VDCC was responsible for the depression of the AHP and for the increase in the firing rate, experiments were performed in the presence of an intracellular calcium chelator (n = 11). To this aim, BAPTA (30 mM), a fast and potent Ca^{2+} chelator, was added to the microelectrode solution and injected into the neuron by applying negative current pulses (2 nA, 20 min). This current dramatically hyperpolarized the neurons (–64 ± 5.7 mV) that were repolarized to their RMP by manually controlling the DC current injected into the cell. BAPTA increased the duration and the frequency of calcium

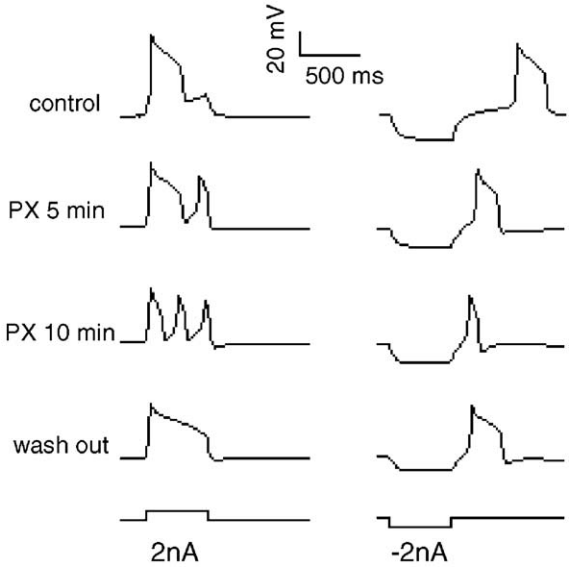

Fig. 2 – Effects of paraoxon on Ca^{2+} spikes evoked by depolarizing current (+2 nA, 500 ms, left panel) and rebound Ca^{2+} spike following hyperpolarizing current (–2 nA, 500 ms, right panel) in the same neuron. Evoked spikes in control, 5 min, and 10 min after paraoxon application and after washout. The trace at the bottom of each panel represents the constant applied current.

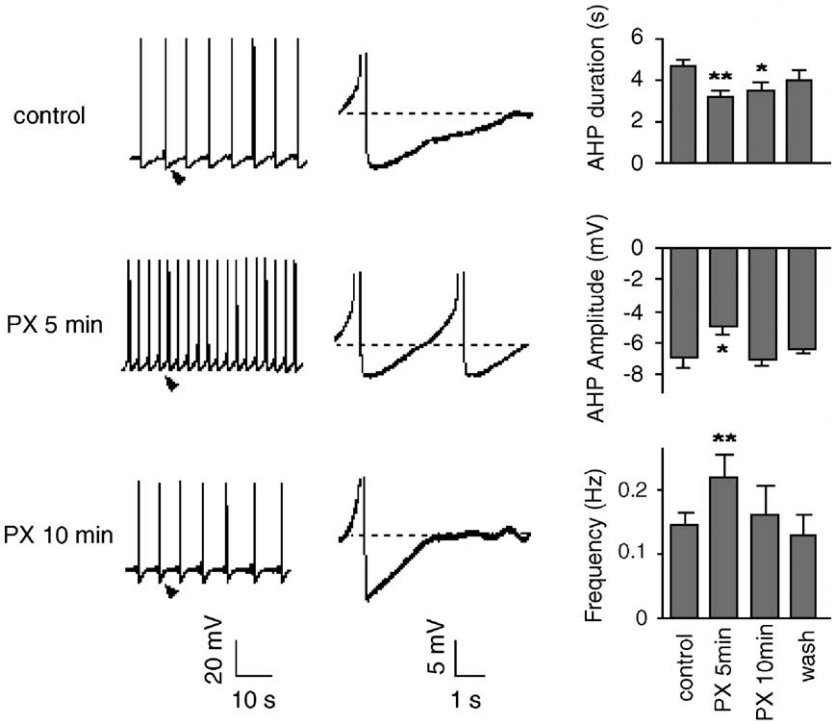

Fig. 3 – Effects of paraoxon on frequency and AHP of spontaneous Ca^{2+} spikes. Left panel: calcium spikes under control condition, 5 min, and 10 min after applying paraoxon. Middle panel: AHPs indicated by arrowhead in higher time and voltage resolution. All traces are from the same neuron, and AP amplitude is truncated in middle panel and dashed line shows the level of RMP. Right panel: Effect of paraoxon on duration of AHP (top), amplitude of AHP (middle), and frequency of Ca^{2+} spikes (bottom) in control, 5 min, and 10 min after exposure to paraoxon and after washout. (n = 17) $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

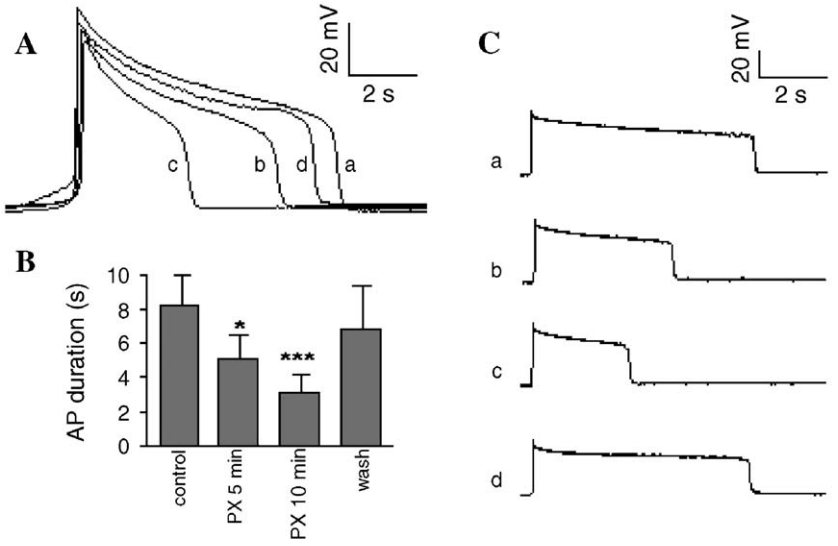

Fig. 4 – Effects of paraoxon on configuration of Ba^{2+} spikes in the absence and presence of ACh receptor antagonists. (A) Spontaneous Ba^{2+} spikes recorded from the same neuron in normal Ba^{2+} Ringer (a), 5 min (b), and 10 min after introducing paraoxon to the preparation and after washout (d). (B) Averaged spike duration obtained from 12 different neurons in Ba^{2+} Ringer in control, paraoxon exposed condition, and after washout. Paraoxon significantly and reversibly decreased the duration of spikes. $^{*}P < 0.05$, $^{***}P < 0.001$ vs. control. (C) Suppression effect of paraoxon on evoked Ba^{2+} spike in the presence of ACh receptor antagonists. Evoked spike after 30-min pretreatment with combined atropine (5 μM) and hexamethonium (50 μM) (a), 5 min (b), and 10 min (c) after application of paraoxon and 10 min after washout with paraoxon-free barium Ringer solution (d). All spikes evoked by depolarizing current (1 nA, 500 ms) in the same neuron and RMP was –45 mV in all traces.

spikes (20.2 ± 4.1% and 43.6 ± 7.5%, respectively; $P < 0.05$) and blocked the AHP (Figs. 5A and B). Application of paraoxon following BAPTA injection produced a 26.8 ± 6.2% decrease in Ca^{2+} spike duration (Fig. 5B).

2.5. *The calcium-activated potassium channel blocker apamin mimics the effect of paraoxon on firing rate*

The effects of apamin pretreatment and subsequent paraoxon application were studied on spontaneously active neurons ($n = 12$). Apamin (1 μM) eliminated a major fraction of AHP by decreasing its duration and amplitude to 36.3 ± 10.3% and 54.7 ± 7.5% of control value, respectively (Fig. 6A). Suppression effect of apamin was accompanied by 132 ± 13% increase in spike frequency (Fig. 6B). Apamin also depolarized the RMP from control value of –41.7 ± 1.7 to –38.5 ± 2.1 mV. The effects of apamin on AHP, frequency and RMP were maximal 12–15 min after its application. Adding paraoxon to the apamin-containing solution led to a 25.2 ± 8.6% decrease in Ca^{2+} spike duration ($P < 0.05$) and a reduction in the amplitude (19.8 ± 5.9%) and duration (16.4 ± 3%) of the AHP (Fig. 6A).

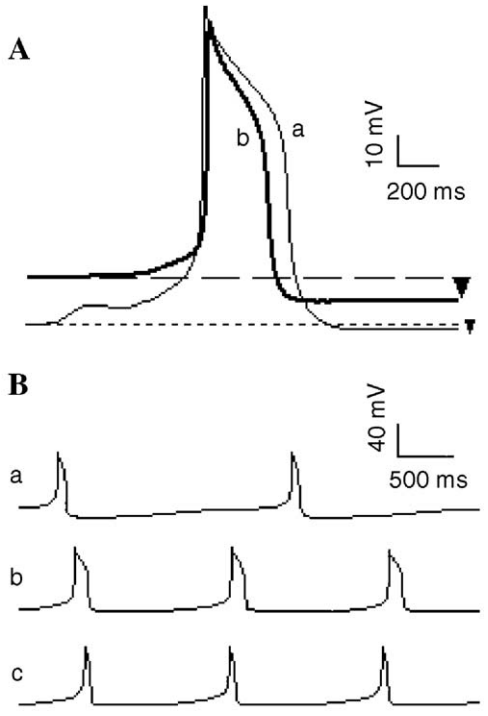

Fig. 5 – Effects of iontophoretically injection of Ca^{2+} chelator BAPTA on Ca^{2+} spikes and modulation of calcium spike configuration by paraoxon following intracellular Ca^{2+} buffering. (A) Superimposed spikes of a neuron in control (a) and 15 min after BAPTA injection (–2 nA, 30 mM) (b). The amplitude of AHP has been shown with larger arrow head in control condition (solid trace) from RMP level (dashed line) to peak of AHP and during BAPTA injection shown by small arrow head from RMP (dotted line) to the peak of AHP. AHP was almost completely abolished by BAPTA injection. (B) Traces of Ca^{2+} spikes in control condition (a), BAPTA injection increased the duration and frequency of spikes (b), paraoxon was able to decrease the duration of spikes after BAPTA injection but did not increase the frequency of spikes (c).

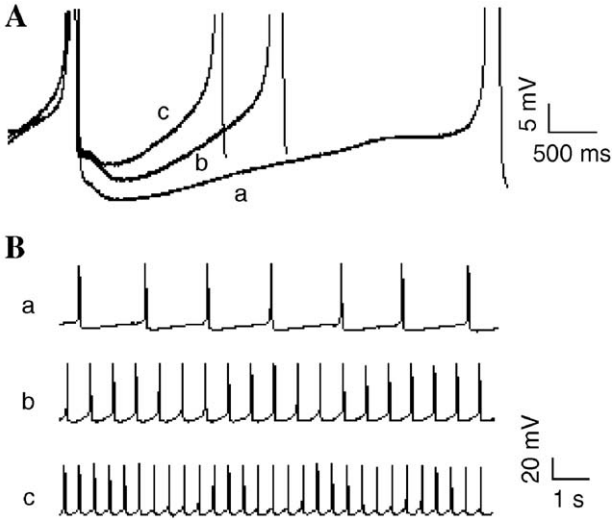

Fig. 6 – Apamin and paraoxon additively decreased duration and amplitude of AHP and increased the frequency of spikes. (A) Superimposed AHPs of a neuron in a high-resolution time and voltage scale in control (a), 15 min after exposure to apamin (b), and 5 min after paraoxon application (c). (B) Trains of Ca^{2+} spikes from the same neuron in control (a), 15 min after applying apamin (b), and 5 min after paraoxon exposure (c).

Moreover, paraoxon further increased the firing rate by 18.2 ± 7.9% (Fig. 6B).

3. Discussion

An increase in the neural excitability is a common effect of acute OPs poisoning (Gunderson et al., 1992; Lebeda and Rutecki, 1985; Lee and Tai, 2001). Although the exact mechanism(s) underlying the OP-induced hyperexcitability is not well known, it is thought to be initiated from an excessive stimulation of nicotinic and muscarinic receptors (Mileson et al., 1998; Sungur and Guven, 2001) which in turn can recruit other neurotransmitter systems (Solberg and Belkin, 1997, McDonough and Shih, 1997). Different ionic currents are known to modulate neuronal firing. Influx of calcium via VDCCs during the action potential leads to an increase in the cytosolic calcium that can initiate a number of physiological processes including activation of Ca^{2+}-dependent K^+ currents. These currents contribute to the action potential repolarization and are largely responsible for the spike frequency modulation (Sah, 1996). In this study, we have found that paraoxon downregulates Ca^{2+} spikes and the following AHP and increases the firing rate in a way that is independent of muscarinic or nicotinic receptor activation.

3.1. *Modulation of Ca^{2+} and Ba^{2+} spikes*

In a Ca^{2+} Ringer, calcium current is the main inward current participating in the depolarization and plateau of the action potentials, which is conducted by VDCCs. Still, modulation of calcium spike by paraoxon may also be exerted by currents

underlying repolarization. A variety of outward potassium currents have been reported in snail neurons. In addition to different kinds of fast and delayed rectifier K^+ outward currents (Bal et al., 2000, 2001), there is another set of potassium channels that are sensitive to intracellular level of Ca^{2+}. Two classes of these channels present in molluscan neurons are large conductance Ca^{2+} mediated K^+ channels (BK channels) and small conductance Ca^{2+} mediated K^+ channels (SK channels). While the first are voltage-dependent and are blocked by TEA, the others are voltage-independent and blocked by apamin (Crest and Gola, 1993; Gola et al., 1990).

Fast (I_A) and delayed outward K^+ currents are blocked with extracellular application of 4-AP and TEA respectively. TEA also blocks the BK calcium-dependent K^+ channels (Kiss et al., 2002; Thompson, 1977, 1982). Therefore, in a calcium Ringer solution, slow calcium-activated K^+ current that passes through SK channels is the main outward current that can modulate the calcium spike configuration (Bal et al., 2001). The suppression effect of apamin, a bee venom toxin selective for certain calcium-activated potassium channels, on AHP also suggests that at least some of these channels are relatively apamin sensitive.

Paraoxon was still able to decrease the duration and amplitude of Ca^{2+} spikes after BAPTA injection and also suppressed the duration of Ba^{2+} spikes. In fact, the balance between calcium and potassium currents will determine the rate of repolarization and therefore the shape (especially the duration) of action potential. The results of the present study suggest that paraoxon can has profound effect on calcium spike shape by blocking the voltage-dependent Ca^{2+} channels. The similar effect of soman, an irreversible cholinesterase inhibitor, on Ca^{2+} and Ba^{2+} spike duration was also reported in bullfrog sympathetic neurons (Heppner and Fiekers, 1991b).

3.2. *Paraoxon induced hyperexcitability*

The anticholinesterase effect of paraoxon on snail neuron has been reported (Filbert et al., 1992; Srivatsan, 1999). Here, submicromolar concentration of paraoxon was used to minimize the AChE inhibition. Furthermore, the increased neural activity was also detected when cholinergic antagonists were present in the bathing medium. This suggests that the possible accumulation of ACh induced by AChE inhibition cannot per se explain the increase in the Ca^{2+} spike frequency.

The increased neuronal activity in the presence of paraoxon is in line with the observed decrease in AHP duration and amplitude (Fig. 4). The AHP that follows action potentials is an important intrinsic negative feedback mechanism controlling excitability. Alterations in the amplitude and duration of AHP have been shown previously to influence neuronal excitability in many different neurons (Goh and Pennefather, 1987; Sah, 1996). A decrease in the duration of AHP increases neuronal excitability (Kawai and Watanabe, 1986; Madison and Nicoll, 1986). The AHP is generated by activation of distinct types of outward K^+ channels by calcium ions entering the neuron during an action potential (Goh and Pennefather, 1987; Vergara et al., 1998). Paraoxon was able to induce a further reduction in AHP duration and amplitude in the presence of apamin along with a significant increase in spike frequency.

Ca^{2+}-dependent K^+ conductances modulate cell firing by contributing toward the RMP, spike repolarization, AHPs and shunting inward currents (Faber and Sah, 2003; Zhang and McBain, 1995; Mills and Pitman, 1999). The duration of AHP, herein, depends on factors which regulate cytosolic calcium concentration. The intracellular calcium required for the AHP in neurons is believed to originate from calcium influx during the action potential, although it can some times be elevated via intracellular stores (Frandsen and Schousboe, 1993; Schousboe et al., 1997; Witt et al., 1994). Decrease in the amplitude and duration of the calcium spikes in the presence of paraoxon, which represents a reduction in calcium entry, may contribute to a decrease in AHP magnitude. It has been shown that VX and soman can reduce the duration of AHP by a decrease in calcium entry during the spike in bullfrog sympathetic neurons (Heppner and Fiekers, 1991a,b, 1992).

The increase in the duration and amplitude of AHPs associated with a decrease in the frequency of spikes, within 10 min of exposure to paraoxon, might be due to a secondary increase in intracellular Ca^{2+}. One possibility is the involvement of intracellular signaling pathways including recruitment of IP_3-sensitive calcium stores as a response to the hyperexcitability (Belan et al., 1990; Kits et al., 1997; Sun et al., 2000) which has been reported in numerous OP-induced status epilepticus (Bodjarian et al., 1993, 1995; Mobley, 1990).

In conclusion, our result confirms and extends previous findings showing that OPs, as a general effect, increase neural firing. We demonstrated that the paraoxon suppression effect on Ca^{2+} spikes in snail neurons may act as a mechanism of induced hyperexcitability. It seems that a reduction in calcium influx through the Ca^{2+} channels and a consequent decrease in AHP duration may participate in paraoxon induced hyperexcitability, suggesting that this mechanism may be involved in the neural excitability associated with OPs intoxication.

4. Experimental procedures

4.1. *Animals and preparations*

Experiments employed 93 specimens of land snail *Caucasotachea atrolabiata* from stocks locally isolated and kept under laboratory conditions. All experiments were performed on neurons in the subesophageal ganglia. The ganglionic mass with its main peripheral nerves and aorta was dissected out and pinned by the nerve and edges of the connective tissue into a small Sylgard-grounded recording chamber with a total volume of 1 ml (Dow Corning Midland, MI, USA). To denude neurons, the connective tissue was gently torn off without any proteolytic enzyme pretreatment.

4.2. *Electrophysiological recording*

Conventional intracellular recordings in current clamp method were performed using the Axoclamp 2B amplifier (Axon instrument, CA, USA). Microelectrodes (Clark instrument, UK) were filled with 3 M KCl and had a resistance of 2–

5 MΩ. In some experiments, BAPTA (30 mM) was added to the intracellular solution. Absolute values of membrane potential were taken as the difference of the stabilized potential 5 min after cell impalement and the zero potential upon withdrawal of the microelectrode from the cell. Neurons with resting membrane potentials exceeding –37 mV, which showed overshooting spontaneous or evoked spikes, were used for analysis.

All experiments were performed at room temperature (21–24 °C). Data were digitized online using a 16-bit A/D converter and stored for further analysis. Membrane R_{in} (in the absence or presence of paraoxon) was calculated from the apparent linear portion of current–voltage relationship obtained by measuring the amplitude of voltage responses to hyperpolarizing current steps (1–5 nA: 500 ms). Ca^{2+} spike was recorded spontaneously or evoked by depolarizing current steps (2 nA, 500 ms) in the absence of extracellular sodium ions (Na^+ ions were replaced on an equimolar basis by TEA). Action potential amplitude and duration were measured from the resting potential to the peak and at half maximal amplitude, respectively. The amplitude of AHP was measured from the resting membrane potential to the peak of AHP, and the duration was measured as the time required declining to 80% of its peak value. In some experiments, where the cells were depolarized (after exposure to paraoxon) or hyperpolarized (after BAPTA injection), RMP was maintained manually close to its control value by a continuous current injection to exclude the possible effect of RMP changes on Ca^{2+} spike configuration and frequency.

4.3. *Solutions and drugs*

The solution used for dissection contained (in mM) NaCl, 84; $CaCl_2$, 10; KCl, 4; $MgCl_2$, 5; glucose, 10; HEPES, 5; pH adjusted to 7.4 with TRISMA-base. Ca^{2+} Ringer was made by substituting NaCl with tetraethyl ammonium chloride (TEA). In some experiments, Ba^{2+}, as a charge carrier, was substituted for Ca^{2+} (Ba^{2+} Ringer). In all experiments, 4-aminopyridine (4 AP, 5 mM), which is generally accepted as a fast outward K^+ channel blocker, was applied to bathing solution.

Paraoxon, *O*,*O*-diethyl *p*-nitrophenyl phosphate (99.8%) was diluted in absolute ethanol to make a 1 M stock solution containing 76.9% ethanol. Dilute aqueous solution of paraoxon (0.3 μM) was prepared daily in Ca^{2+} or Ba^{2+} Ringer. Final concentration of ethanol in superfusing solution was less than 0.01%. The same concentration of ethanol had no effect on Ca^{2+} spikes (Janahmadi et al., 1995). 1,2-*bis*(2-Aminophenoxy) ethan-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) (30 mM) was injected iontophoretically with hyperpolarizing pulses (2 nA, 20 min). Paraoxon, TEA, HEPES, 4-AP, atropine, hexamethonium, and BAPTA were purchased from Sigma (St. Louis, MO, USA). Other chemicals were obtained from Merck (Darmstadt, Germany).

Numerical results are given as mean ± SEM with *n* being the number of cells on which the measurements were done. Significant differences were evaluated by paired *t* test and ANOVA followed by Tukey's test with the level of significance set at $P < 0.05$.

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