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ScienceDirect
NeuroToxicology 28 (2007) 1178–1183
NeuroToxicology

Forskolin potentiates the paraoxon-induced hyperexcitability in snail neurons by blocking afterhyperpolarization

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Received 8 June 2007; accepted 11 July 2007
Available online 17 July 2007

Abstract

One characteristic of organophosphate poisoning is the ability to increase excitability or induce epileptiform activity in nerve cells, but underlying mechanisms are not fully understood. We have previously reported that paraoxon, an organophosphate compound, at submicromolar concentrations effectively suppress Ca^{2+} spikes and modulate the activity of snail neurons. This effect was unrelated to acetylcholinesterase (AChE) inhibition but was found to involve the direct or indirect modulation of ion channels [Vatanparast J, Janahmadi M, Asgari AR, Sepehri H, Haeri-Rohani A. Paraoxon suppresses Ca^{2+} spike and afterhyperpolarization in snail neurons: relevance to the hyperexcitability induction. Brain Res 2006a;1083(1):110–7]. In the present study, the interaction of paraoxon with cAMP formation on the modulation of Ca^{2+} spikes and neuronal excitability was examined. Forskolin, the activators of adenylate cyclase, suppressed afterhyperpolarization (AHP) and increased the activity of snail neurons without any significant effect on the Ca^{2+} spike duration. Pretreatment with forskolin, although attenuated the suppressing effect of paraoxon on the duration of Ca^{2+} spikes but also potentiated the paraoxon-induced hyperexcitability by enhancing the suppressive effects of paraoxon on AHP. Our findings support the possible involvement of cAMP formation in the paraoxon-induced AHP suppression and neuronal hyperexcitability, although activation of cAMP pathway may attenuates some effects of paraoxon.

Keywords: Paraoxon; Forskolin; Snail neuron; Hyperexcitability; Afterhyperpolarization

1. Introduction

Organophosphorus compounds used as insecticides include various types of compounds such as phosphates, phosphorothionates, and phosphorothiolates. Despite waning household uses, OPs are still used widely as general purpose insecticides, agricultural and horticultural pesticides, veterinary medicines and also on a variety of foods and commercial crops.

Neural hyperexcitability is a frequently reported symptom of acute poisoning with organophosphates (Ohbu et al., 1997; Shih and McDonough, 1999). OP insecticides (e.g., paraoxon) exert their neurotoxicity by irreversibly inhibiting AChE, leading to ACh accumulation and overstimulation of cholinergic pathways (Taylor, 1996).

However, there are evidences that paraoxon can interact with targets (e.g., receptor and voltage gated ion channels) other than AChE in vertebrate and invertebrate neuronal soma membrane that could potentially affect the neuronal firing by modulating the membrane ionic channel currents (Filbert et al., 1992; Rocha et al., 1996).

The interaction of some organophosphates (OPs) with membrane Ca^{2+} channels has been reported (Heppner and Fiekers, 1991; Vatanparast et al., 2006a,b). Biochemical and electrophysiological approaches have also addressed OP modulation of some metabotropic receptors (in particular muscarinic type) and downstream elements (Bakry et al., 1988; Katz and Marquis, 1989; Silveira et al., 1990; Sun et al., 2000; Ward and Mundy, 1995). Signal transduction cascades activated by metabotropic receptors can underlie transient modulation of intracellular Ca^{2+} concentration via phosphorylation or dephosphorylation of Ca^{2+} channels (Catterall, 1997, 2000; Rossie, 1999; Strock and Diversé-Pierluissi, 2004;

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0161-813X/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.neuro.2007.07.003

Swartz et al., 1993; Zhou and Ikeda, 1994), and/or by activating intracellular messenger cascades that lead to Ca^{2+} mobilization from intracellular stores. For example activation of muscarinic receptors modulates variety of L-, N- and P-type Ca^{2+} channels via different pathways (Howe and Surmeier, 1995; Wicher, 2001). A number of studies also have reported that some OP toxicants can interact directly with muscarinic receptors (Eldefrawi and Eldefrawi, 1983).

The goal of the present study was to test the hypothesis that activation of cAMP formation is involved in neuronal hyperexcitability induced by paraoxon. For this purpose, we sought to examine the singular effects and the interplay of forskolin, a direct activator of adenylate cyclase, and paraoxon on neuronal excitability in snail neuron.

2. Materials and methods

Experiments were performed on central neurons in the subesophageal ganglia of land snail *Caucasotachea atrolabiata*. The circum-oesophageal ganglia were dissected out and were fixed dorsal side up on a Sylgard coated recording chamber (Dow Corning Midland, MI, USA) in normal snail Ringer. The composition of normal snail Ringer was (in mM): NaCl, 84; $CaCl_2$, 10; KCl, 4; $MgCl_2$, 5; glucose, 10; HEPES, 5 (pH 7.4). To expose neurons, the connective sheathes were mechanically torn using fine forcipes without any pretreatment with proteolytic enzymes. These procedures were in accordance with the guidelines of the Institutional Animal Ethics Committee at Shaheed Beheshti Medical Sciences University. To elicit Ca^{2+} spikes the extracellular medium was changed by a circulating Ringer in which the NaCl content of normal snail Ringer was replaced by TEA, and to which 4-aminopyridine (4-AP, 5 mM) was added (Ca^{2+} Ringer). All experiments were performed at room temperature (21–24 °C).

Microelectrodes (Clark Instrument, UK) were filled with 3 M KCl and those with a resistance of 2–5 MΩ were used for recording. The reference electrode was an agar bridge containing normal Ringer and 3 M KCl in series which was connected to earth via an Ag–AgCl wire. Intracellular recordings were obtained in current clamp mode using an Axoclamp 2B amplifier. The recorded data were digitized using an A/D converter (AD Instrument, Australia) and saved on computer for offline analysis. The parameters of spontaneously recorded Ca^{2+}spikes and following AHPs, duration and amplitude, were measured using Chart 5 software as described before (Vatanparast et al., 2006a). Data were presented as means ± S.E.M. with *n* being the number of neurons on which the measurements were done. The statistical differences were determined by the Student's *t*-test and ANOVA followed by Tukey's multiple comparison test with a significance level of 0.05.

Paraoxon, *O*,*O*-diethyl-*p*-nitrophenyl phosphate (Sigma, USA) was prepared as 1 M stock in absolute ethanol and diluted (0.3–0.6 μM) daily with Ca^{2+} Ringer. In some experiments ganglia were pretreated in a Ca^{2+} Ringer containing atropine sulphate (Sigma, 5 μM) to block the muscarinic receptors. Forskolin was added to bath medium from stock solution in dimethyl sulphoxide (DMSO). Final DMSO concentration was <0.01%.

3. Results

Experiments were made on 46 neurons that showed spontaneous firing in calcium Ringer containing voltage dependent potassium channel blockers (4-AP and TEA). In control conditions, the recorded spontaneous Ca^{2+} action potentials had a mean duration of 0.28 ± 0.06 s, AHP of 4.43 ± 0.22 s and a frequency of 0.143 ± 0.02 Hz ($n = 21$).

Paraoxon suppressed the duration of Ca^{2+} action potentials in a time dependent manner. Application of paraoxon (0.3 μM) within 10 min induced a reduction of 34.7 ± 7.2% in the duration of spikes. Paraoxon also induced a reduction of 28.6 ± 8.1 and 21.4 ± 9.4% in the duration of AHP within 5 and 10 min of application, respectively, which was along with an increase in the frequency of firing (Fig. 1). The maximum frequency was observed 5 min after paraoxon application by 52.5 ± 16.4% increase compared to control condition ($P < 0.05$). In the previous study we showed that a direct blockade of Ca^{2+} channels participates as a mechanism of paraoxon-induced hyperexcitability via downregulation of Ca^{2+} dependent K^+ channels that underlies AHP. We also found that within 10–15 min of exposure to 0.3 μM the duration of AHP showed a secondary increase associated with a

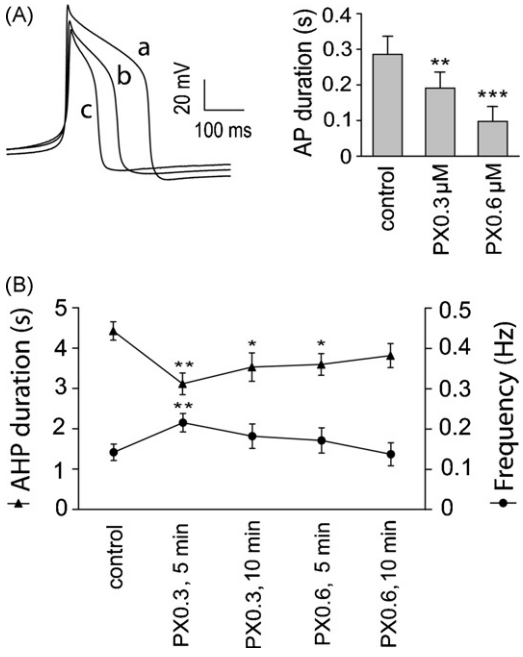

Fig. 1. Paraoxon (PX) decreased the duration of Ca^{2+} spikes and had a dual effect on the duration of AHP and firing rate. (A) Superimposed Ca^{2+} spikes recorded from a neuron in normal Ca^{2+} Ringer (a), 10 min after exposure to 0.3 μM paraoxon (b) and 10 min after application of higher concentration of paraoxon (0.6 μM) (c). Bar histogram shows the effects of 10 min of exposure to the 0.3 μM and 0.6 μM paraoxon on the mean duration of Ca^{2+} spikes. (B) A graph representing the mean frequency of Ca^{2+} spikes and mean duration of AHP in control condition, 5 and 10 min after application of 0.3 μM paraoxon and also 5 and 10 min after increasing the concentration of paraoxon to 0.6 μM ($n = 11$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control.

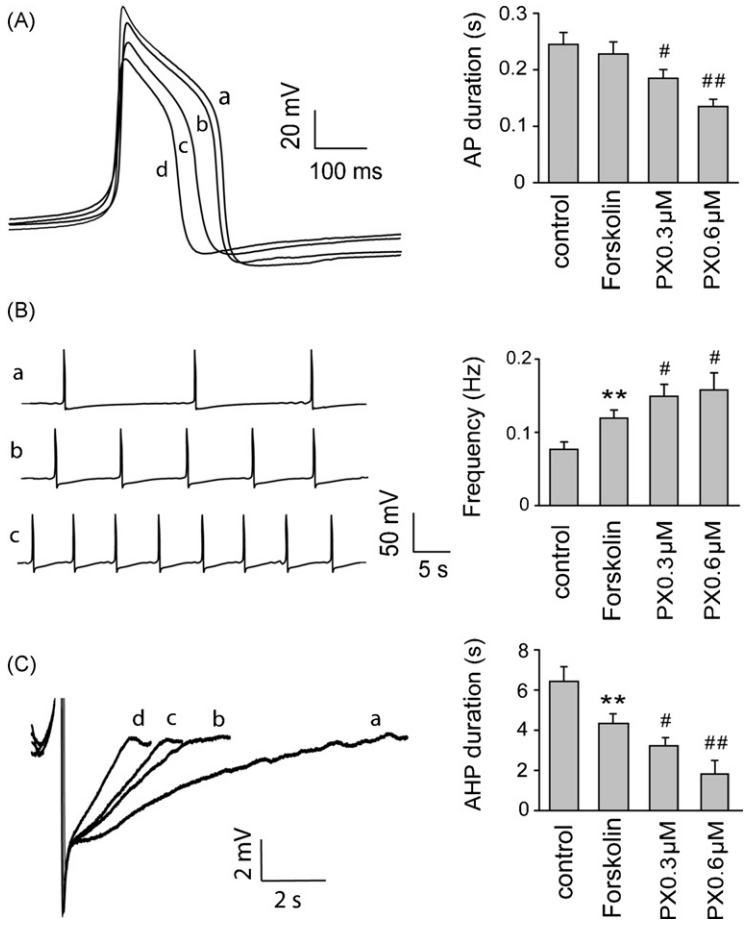

Fig. 2. The effects of forskolin and the following application of paraoxon on the duration of Ca^{2+} spikes, frequency of spikes and duration of AHP. (A) Spontaneous Ca^{2+} spikes from a neuron in control condition (a), 15 min after application of 25 μM forskolin (b), 10 min after application of paraoxon (0.3 μM) to the forskolin containing extracellular medium (c) and 10 min after increasing the concentration of paraoxon (to 0.6 μM) (d). (B) Trains of spontaneously recorded Ca^{2+}spikes from a neuron in control condition (a) 15 min after application of forskolin (b) and 10 min after application of paraoxon (0.3 μM) to the forskolin containing Ringer (c). (C) superimposed truncated Ca^{2+} spikes from a neuron that show AHPs following Ca^{2+} spikes in control condition (a), 15 min after application of forskolin (b), 10 min after exposure to 0.3 μM paraoxon (c) and 0.6 μM paraoxon (d) in the presence of forskolin. Histograms represent the effects of forskolin and 10 min of exposure to 0.3 and 0.6 μM paraoxon on the duration of Ca^{2+} spike, frequency and AHP duration ($n = 14$). $^{**}P < 0.01$ vs. control and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. forskolin treated condition.

decrease in frequency and this was despite of ongoing suppression of Ca^{2+} spikes (Vatanparast et al., 2006a). Application of higher concentration of paraoxon (0.6 μM) within 10 min caused a reduction of 65.72 ± 6.71% in the duration of spikes (Fig. 1A). Although increasing the concentration of paraoxon to 0.6 μM produced further decrease in the duration of spikes but it led to an increase in the duration of AHP along with more suppression in the firing rate (Fig. 1B).

3.1. The effects of forskolin and following paraoxon exposure on Ca^{2+} spikes configuration and frequency

To study the effects of cAMP formation on Ca^{2+} spikes and neuronal firing and to find out its possible interaction with paraoxon, the ganglia were superfused with Ca^{2+} Ringer containing 25 μM of forskolin as a direct adenylate cyclase activator. Forskolin within 15 min of application did not significantly change the duration of Ca^{2+} spikes. However, exposure to paraoxon (0.3 μM) in the presence of forskolin induced a reduction of 21.48 ± 6.3% in the duration of spikes. By application of higher concentration of paraoxon (0.6 μM) this reduction reached to 38.89 ± 5.8% within 10 min (Fig. 2A).

In addition forskolin within 15 min induced an increase of 48.75 ± 9.35% in the frequency of spikes and a decrease of 32.6 ± 11.2% in the duration of AHP. Paraoxon (0.3 μM) in the presence of forskolin caused a further increase of 25.7 ± 7.4% in frequency of spikes and a decrease of 19.5 ± 5.4% in the duration of AHP. Within 10 min of increasing the concentration of paraoxon to 0.6 μM an increase of 33.8 ± 8.1% in the frequency of spikes and a decrease of 41.9 ± 14.7% in the duration of AHP, compared to forskolin treated condition, was recorded (Fig. 2B and C). Both forskolin and paraoxon-induced hyperexcitability were associated with a decrease in duration of AHP. The duration of AHPs was decreased in the presence of forskolin by 32.8 ± 9.4% compared to control conditions. Exposure to 0.3 and 0.6 μM paraoxon, respectively, induced 19.5 ± 5.4 and 41.9 ± 14.7% decrease in duration of AHP compared to forskolin treated condition.

4. Discussion

Paraoxon, the active metabolite of parathion, is a potent inhibitor of AChE (Atterberry et al., 1997; Mortensen et al., 1998). Parathion is one of the most acutely toxic pesticides registered by the EPA (environmental protection agency). Because of its high toxicity and risks of exposure to agricultural workers and to birds the use of parathion on fruit, nut and vegetable crops is prohibited in most of countries. The only uses retained are those on alfalfa, barley, corn, cotton, sorghum, soybeans, sunflowers and wheat. Further, to reduce exposure of agricultural workers, parathion may be applied to these crops only by commercially certified aerial applicators and treated crops may not be harvested by hand.

While inhibition of AChE initiates a well-established mechanism of cholinergic toxicity, a number of surveys have reported additional ''noncholinesterase'' actions for OP anticholinesterases that could be of toxicological relevance (Volpe et al., 1985; Silveira et al., 1990; Pruett et al., 1994; Richards et al., 2000).

The present study demonstrated that the modulation of neuronal activity by paraoxon is potentiated by PKA activation. We have previously described that during acute exposure to low concentration of paraoxon in the presence of specific muscarinic and nicotinic antagonists, atropine (5 μM) and hexamethonium (50 μM), a decrease in Ca^{2+} influx and associated suppression of AHP could provide a basis for primary paraoxon-induced hyperexcitability (Vatanparast et al., 2006a). However, an important question remains about the intracellular signals that involve in neuronal firing alteration in the presence of paraoxon.

Phosphorylation-dependent ion channel regulation is a mechanism for modulation of neuronal excitability (Conn et al., 1989a,b; Hille, 2001; Levitan and Kaczmarek, 2002; Magoski, 2004). Different kinases and phosphatases that mediate this regulation have been found closely associated with particular ion channels, especially Ca^{2+} and Ca^{2+} dependent K^+ channels that are critical contributors to the neural firing behavior (Bielefeldt and Jackson, 1994; Chung et al., 1991; Faber and Sah, 2003; Rosenmund et al., 1994; Reinhart and Levitan, 1995; Scuri et al., 2005; Zhou et al., 2002). Some ion channels have intrinsic sites for binding of Ca^{2+} and/or calmodulin on their intracellular surface, so that intracellular Ca^{2+} have direct effects on the gating of these channels (Hille, 2001).

4.1. Possible contribution of cAMP formation on the suppressing effect of paraoxon on Ca^{2+} spikes

Forskolin did not significantly change the duration of Ca^{2+} spikes, consistent with the literature (Paupardin-Tritsch et al., 1996). Paraoxon decreased the duration of Ca^{2+} spikes in the presence of forskolin, the PKA activators, but this reduction was considerably less than when ganglia were exposed to paraoxon alone. The reduction in the duration of Ca^{2+} spikes within 10 min of exposure to 0.3 and 0.6 μM paraoxon alone was 35% and 63%, respectively. However, in the presence of forskolin the decrease in the duration of Ca^{2+} spike by these concentrations of paraoxon was 21% and 39%, respectively. These finding shows that the activation of cAMP formation attenuates the suppressing effect of paraoxon on the duration of Ca^{2+} spikes. The effect of cAMP pathway and PKA on Ca^{2+} currents has been reported in different neurons (Conn et al., 1989a,b; Catterall, 2000). In F_1 neurons of *Helix aspersa* has been shown that catalytic subunit of PKA reverse the rundown of Ca^{2+} current that happens in the dialyzed neuron under whole cell clamp, while the blockade of protein phosphates increase the Ca^{2+} current (Golowasch et al., 1995). The increment in the Ca^{2+} current in the presence of PKA activator has been attributed to the modulatory effect of PKA on Ca^{2+} channel by phosphorylation that increases their conductance (Hille, 2001).

4.2. The modulatory effect of forskolin on neuronal activity and interaction with low concentrations of paraoxon

Forskolin increased the frequency of Ca^{2+} spikes along with a decrease in the duration of AHP. The excitatory effect of PKA activator on different neurons, including snail neurons, have already been reported (Conn et al., 1989a,b; Hille, 2001; Zhang et al., 2001). Some neurotransmitters (e.g., monoamines and acetylcholine) decrease the spike frequency adaptation via metabotropic receptors in hippocampal neurons. This process involves attenuation of the SK channel mediated current, which in turn suppresses sI_{AHP}. As a result, AHP is sharply reduced and causes the cell to fire repetitively (Hille, 2001; Krause and Pedarzani, 2000). The effect of monoamines is mimicked by cAMP analogous or by stimulating adenylyl cyclase with forskolin (Pedarzani and Storm, 1993, 1995). In contrast, the cholinergic suppression of sI_{AHP} appears to be independent of PKA (Blitzer et al., 1994; Pedarzani and Storm, 1993) and involves a G-protein-mediated protein phosphatase (Krause and Pedarzani, 2000). In snail neurons a direct inhibition of K^+ channels by stimulating the adenylate cyclase activity has also been reported (Deterre et al., 1982; Watanabe and Gola, 1987).

We found that paraoxon can increase the frequency of action potentials in the presence of forskolin. Previous studies have reported the mechanisms by which the activation of PKA can facilitate membrane excitability in different types of tissues. Song et al. (2006) reported that cAMP contributes to sensory neuron hyperexcitability in rats. In enteric AH neurons have also been shown that cAMP formation is associated with suppression of the slow AHP and an increase in the excitability (Vogalis et al., 2003).

In our previous work we showed that exposure to submicromolar concentrations of paraoxon may directly affect membrane excitability via suppression of Ca^{2+} entry during the action potential, which would down regulate the Ca^{2+}-activated K^+ channels and leading to a reduction in the AHP and an increase in cell firing (Vatanparast et al., 2006a). On the other hand, here in forskolin treated neurons, application of paraoxon led to a robust neuronal hyperexcitability.

From the above results, it might be suggested that increased intracellular cAMP, following application of forskolin, might

modulate the release of Ca^{2+} from IP_3-sensitive Ca^{2+} store to reduce the Ca^{2+}-activated K currents and thereby facilitates the neuronal excitability in the presence of paraoxon. We have recently shown that pretreatment with 8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8), an antagonist of IP_3-mediated Ca^{2+} release, abolished the secondary silencing effect of paraoxon, which is observed after primary paraoxon-induced hyperexcitability (Vatanparast et al., 2007).

It has also been reported that in molluscan neurons an increase in the intracellular concentration of cAMP can modify voltage-dependent K currents that contribute to spiking activity (Alkon et al., 1983; Castdllucci et al., 1980; DePeyer et al., 1982; Kaczmarek and Strumwasser, 1984) and ionic currents that are active at subthreshold voltages and influence the repetitive firing characteristics of the neurons (Aldenhoff et al., 1983; Connor and Hockberger, 1984; Deterre et al., 1982; Drummond et al., 1980; Green and Gillette, 1983; Pellmar, 1981; Siegelbaum et al., 1982). Forskolin, a lipid soluble diterpene extracted from Coleus forskolii, is potentially an important tool for studying the modulation of ionic currents and neuronal excitability by cAMP because it stimulates adenylate cyclase in a variety of cells, including molluscan neurons (Deterre et al., 1982; Kauer and Kaczmarek, 1985; Seamon and Daly, 1981; Seamon et al., 1981). Deterre et al. (1982) showed that both forskolin and cAMP injection depress an outward K^+ current in Helix neurons. Voltage-clamp studies of the transient K^+ current, I_A, and the voltage-dependent delayed K^+ current, IK, in neuroendocrine cells of *Aplysia* showed that forskolin and cAMP modify these currents in similar ways (Strong, 1984; Strong and Kaczmarek, 1986). Another possible explanation might be the depression of fast I_A channel current which is sensitive to forskolin (Furukawa et al., 1992), so that inhibition of this outward K^+ channel current resulted in the reduction of the AHP duration and subsequent increase in the neuronal excitability (Sah and McLachlan, 1992).

Taken together, these finding suggests that forskolin might potentiate the blocking effect of paraoxon on afterhyperpolarization and thereby affecting the excitability.

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