



Modulation of dopaminergic neurotransmission induced by sublethal Doses of the organophosphate trichlorfon in cockroaches



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ABSTRACT

Organophosphate (OP) insecticides have been used indiscriminately, based on their high dissipation rates and low residual levels in the environment. Despite the toxicity of OPs to beneficial insects is principally devoted to the acetylcholinesterase (AChE) inhibition, the physiological mechanisms underlying this activity remain poorly understood. Here we showed the pharmacological pathways that might be involved in severe alterations in the insect locomotion and grooming behaviors following sublethal administration of the OP Trichlorfon (Tn) (0.25, 0.5 and 1 μM) in *Phoetalia pallida*. Tn inhibited the acetylcholinesterase activity (46 ± 6 , 38 ± 3 and 24 ± 6 nmol NADPH/min/mg protein, $n=3$, $p < 0.05$), respectively. Tn (1 μM) also increased the walking maintenance of animals (46 ± 5 s; $n=27$; $p < 0.05$). Tn caused a high increase in the time spent for this behavior (344 ± 18 s/30 min, 388 ± 18 s/30 min and 228 ± 12 s/30 min, $n=29-30$, $p < 0.05$, respectively). The previous treatment of the animals with different cholinergic modulators showed that pirenzepine > atropine > oxotremorine > *d*-tubocurarine > tropicamide > methoctramine induced a decrease on Tn (0.5 μM)-induced grooming increase, respectively in order of potency. Metoclopramide (0.4 μM), a DA-D₂ selective inhibitor decreased the Tn-induced grooming activity (158 ± 12 s/30 min; $n=29$; $p < 0.05$). Nevertheless, the effect of the selective DA-D₁ receptor blocker SCH 23390 (1.85 μM) on the Tn (0.5 μM)-induced grooming increase was significant and more intense than that of metoclopramide (54 ± 6 s/30 min; $n=30$; $p < 0.05$). Taken together the results suggest that a cross-talking between cholinergic M1/M3 and dopaminergic D1 receptors at the insect nervous system may play a role in the OP-mediated behavioral alterations.

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1. Introduction

Organophosphate insecticides (OP) derived from phosphoric or thiophosphoric acid include parathion, malathion, diazinon, fenthion, chlorpyrifos (Nishizawa 1960; Fest and Schmidt 1982; Medegela et al. 2010), and trichlorfon (Tn) among others. Tn (metrifonate or dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate) inhibits acetylcholinesterase (AChE) due to its ability to phosphorylate its serine hydrolase at its esterase active site. AChE inhibition leads to accumulation of acetylcholine at the cholinergic junction from nerve tissue and effector organs, producing acute

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effects at the muscarinic and nicotinic receptors and at the central nervous system (CNS) (Torres-Altora et al. 2011; Cummings and Ringman 1999). Tn was first registered in the United States in 1955, and nowadays is a worldwide diffused systemic insecticide with a variety of domestic and agricultural applications (EPA 2006). Li and colleagues (2011) (Li et al., 2011) have shown that the dissipation rates of Tn on soil and cabbage are about 90% in five days, suggesting that Tn is safe when applied at the recommended dosage. Although the dissipation and degradation rates of OP are high, the potential effect of residual amounts of these insecticides in the surrounding environment is still a matter of concern. In this way, even if their concentrations are low in the environment (Gupta et al., 2012), the reminiscent activity of these insecticides are amenable to induce long-term behavioral alterations in insects (de Castro et al., 2013; He et al., 2013; Neuman-Lee et al., 2013).

During the last two decades, the effects of OP on beneficial arthropods have been the subject of an increasing number of

studies, and their potential effects have been reviewed several times (Croft, 1990; Haynes, 1988; Thompson, 2003; Desneux et al., 2007). In addition to the direct mortality, the effects of sublethal doses of OP on arthropod physiology and behavior must be considered in a complete analysis of their impact, although these behavioral alterations are difficult to study or predict (Desneux et al., 2007). While evaluations associated with the direct effects of pesticides on natural enemies are important, the indirect or delayed effects of pesticides are actually more relevant because they provide information on the long-term stability and overall success of a biological control program when attempting to integrate the use of pesticides with natural enemies (Cloyd, 2012). Therefore, these behavioral alterations could increase the chances of beneficial insects being preyed upon, and thus have the potential for serious ecological consequences through trophic transfer.

Early studies of Guez and colleagues (2005) have shown important alterations induced by sublethal doses of OPs on foraging behavior in honeybees. Castro et al. (2013) and Campos et al. (2001) also have described the effect of sublethal concentrations of OPs on mobility parameters in pentatomid stinkbugs, a natural predator of defoliating caterpillars, and in earwigs, a predator of armyworms, respectively. Although the authors have identified the behavioral alterations derived from the insect OP sublethal intoxication, none of these studies have deeply addressed specific neurophysiological mechanisms. In this context, any indirect effects, which are sometimes referred to as sub-lethal, latent, or cumulative adverse effects may interfere with the physiology and behavior of natural enemies by inhibiting longevity, fecundity, reproduction, development time, behavior (mobility, searching, foraging, and feeding), predation and/or parasitism, prey consumption, emergence rates, and/or sex ratio (Cloyd, 2012).

Despite the role of dopamine in modulating behavior in insects has been extensively studied, the influence of cholinergic modulators (e.g. sublethal doses of OP) on this parameter is still not well understood. Furthermore, most neurons in the nervous system apparently contain and release more than one neurotransmitter or neuromodulator. Because of this, it is thought that such co-transmission should be considered as a rule instead of an exception (Trudeau, Gutierrez, 2007). In this aspect, cockroaches are primitive insects in which most of functional systems are fairly unspecialized and for this reason have been used not only to understand the physiological aspects between insect and chemical compounds, but also as an important model for biomedical research (Stankiewicz et al. 2012).

Here we sought to investigate the role of sublethal doses of Tn in modulating biochemical and neurophysiological parameters affecting insect behavior, using cockroaches (*Phoetalia pallida*). We have also compared the in vitro and in vivo effects of Tn on the cholinergic and dopaminergic systems of the insects. However, as far as of our knowledge, there is no previous work demonstrating the extra-cholinergic pathway involved in the modulation of insect behavior by organophosphates. Thus, the rationale for such study in cockroaches is not restricted to the pharmacology of OP itself, but may contribute for the elucidation of the unknown mechanisms involved in the recent behavioral alterations of other beneficial insects like honey bees.

2. Materials and methods

2.1. Experimental animals

All experiments were performed on adult male *P. pallida* cockroaches (3–4 months after adult molt). The animals were reared at laboratory conditions of controlled temperature (22–25 °C) on a 12 h:12 h L:D cycle. All cockroaches were provided with water and dog food ad libitum. Prior to analysis of biochemical and

neurophysiological parameters, the maximum sublethal dose of Tn was determined in cockroaches essentially as described by Kagabu et al. (2007).

2.2. Drugs and chemicals

Tn, atropine, tyramine, methoclopramide, pyrenzepine, methocramine, d-tubocurarine, oxothremorine, tropicamide, nifedipine, and SCH23-390 were obtained from Sigma-Aldrich (USA) and were freshly prepared in HEPES saline of the following composition (in mMol l⁻¹): NaCl, 214; KCl, 3.1; CaCl₂, 9; sucrose, 50; HEPES buffer, 5 and pH 7.2 (Wafford and Sattelle 1986). All drugs were administered at the third abdominal hemocoel segment, at a final volume of 20 µL by means of a Hamilton syringe. Experiments were performed at controlled room temperature (22–25 °C) in accordance with the guidelines of the National Counselor to Control Animal Experimentation-CONCEA.

2.3. Assay for determining sublethal doses

The insecticidal assay against adult *P. pallida* was conducted in an adaptation of the assay described by Kagabu et al. (2007). The average weight of the insects (MW) was determined by weighting three hundred adult animals. Thus, the MW was found to be 0.55 g, which was used to calculate the actual doses to be used. Tn was dissolved in HEPES saline and injected into the second abdominal segment of *P. pallida* at concentrations ranging from 0.25 to 4 µM, in a final volume of 20 µL by means of a Hamilton syringe. Three insects were used to test each dose of trichlorfon and after treatment they were kept in a plastic bottle at 22–25 °C for 24 h with free access to water and food. The minimum dose that killed all the three insects in 24 h was considered as the minimum lethal dose (MLD). Paralyzed insects were also considered as if they were dead. In our experimental condition, it was found that the MLD was 2 µM, and the 4 µM concentration was able to kill all the three animals in 24 h. Thus, in the present work doses below the MLD were chosen for conducting the behavioral assays.

2.4. Assay for cholinesterase activity

The in vitro activity of AChE was evaluated according to the assay described by Ellman et al. (1961) modified by Franco et al. (2009). The whole amount of protein was measured according to Bradford (1976). Three cockroaches were injected with Tn (0.25, 0.50, and 1 µM) and after 6 h were anesthetized by chilling at –5 °C and their brains were collected following the removal of the cuticle. The tissue was mixed with 750 µL of Kpi buffer pH 7.0, centrifuged (500 rpm/5 min/4 °C) and 400 µL of supernatant was collected. Fifty µL of the supernatant was added to 50 µL of 50 mM DTNB, 500 µL of Kpi (pH 8.0) and 2.5 µL of acetylthiocholine. The reaction was measured after 1 min at 412 nm using a UV-Visible Spectrophotometer (model Evolution 60 S, Thermoscientific, New Hampshire, USA). The results were analyzed by the software VISION lite (Thermoscientific).

2.5. Video-mounting apparatus for biological assays

For each specific biological assay, the activities were recorded during 30 min by using a video-camera (Panasonic coupled to a 50 mm Karl-Zeiss lens) connected or not to an eyepiece of microscopy (Olympus, model SZ51, Germany). The camera had a frame-by-frame (60/s) and was connected to a PC (Infoway, ItauTec, Brazil). Video movies were later analyzed using a HD Writer AE 2.6 T system (Panasonic) with variable speed control.

2.6. Biological assays

2.6.1. Behavioral assays

For general behavioral study, animals were placed in a demarked open-field arena with a video camera mounted overhead.

2.6.1.1. Sustained locomotor activity. In order to analyze walking maintenance, a behavior paradigm based on the methodology described by Gal and Libersat (2008), namely the forced swimming test was applied. A continuous stressful stimulus producing strong, stereotypic aversion responses was created by water immersion (Cocatre-Ziligien and Delcomyn 1990; Gal and Libersat 2008). Swimming in a modified forced swimming test was induced by placing the cockroaches in an opaque pool (25 cm in diameter) filled with water to a height of 10 cm, maintained at 25 °C. The insects were monitored for 1 min period using a video camera. The swimming behavior was based on the time the insects kept in motion.

2.6.1.2. Grooming activity. The grooming behavior of cockroaches was monitored in an opaque plastic box (29 cm x 18 cm x 13 cm) with a clear plastic cover (Weisel-Eichler et al. 1999) and was recorded with a camera for later analysis of motion time. The time of continuous grooming in seconds was measured for a 30 min

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