Pesticide Biochemistry and Physiology 100 (2011) 35–40

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Effects of pyrethroids on neuronal excitability of adult honeybees *Apis mellifera*

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ARTICLE INFO

Article history:
Received 13 July 2010
Accepted 13 February 2011
Available online 23 February 2011

Keywords:
Pyrethroid
Honeybee
Whole-cell patch-clamp
Neuron
Sodium current

ABSTRACT

Pyrethroids act on the nervous system as a primary target organ and exert their neurotoxic effects primarily by altering the conductance of sodium channel, leading to hyperexcitation. However, few studies investigated the effects of pyrethroids on neuronal excitability of honeybee brain neurons. In this study, a whole-cell patch-clamp technique was used to record current threshold, the minimum current to induce an action potential, and peak sodium current in the dissociated honeybee brain neurons treated with bifenthrin, deltamethrin and fluvalinate in vitro & in vivo. The study showed that these pyrethroids greatly suppressed the neuronal excitability as revealed by increasing current injected and inhibited the peak sodium current in honeybees. The three pyrethroids also inhibited steady-state inactivation in addition to reduction of sodium peak current.

1. Introduction

Pyrethroids are synthetic derivatives of pyrethrins and are widely used in agriculture to control insect pests due to their high insecticidal potency, low mammalian toxicity, and biodegradability. Honeybees, when collecting nectar from the flowers of those plants treated with pesticides, will come in contact with pyrethroids. In addition, a pyrethroid pesticide tau-fluvalinate is used to control parasitic mites inside honeybee colonies [1]. Therefore, widespread use of pyrethroids has increased the likelihood of poisoning on honeybees. The honeybee is extremely susceptible to insecticides as its genome has fewer genes encoding xenobiotic detoxifying enzymes compared to other insects [2]. It has been demonstrated previously that honeybees exposed to pyrethroid pesticides exhibited an impairment of colony vitality [3], reduced acetylcholinesterase activity [4,5], and poorer foraging and olfactory learning performance [6]. In the present study, we found that queens of honeybee colonies fed with bifenthrin (but not deltamethrin or fluvalinate) lay multiple eggs in one cell, in comparison with one egg per cell in the control.

Pyrethroids are neurotoxins that alter the normal function of insect nerve system [7]. The principal sites of insecticidal action of pyrethroids are neuronal receptors and/or ion channels, thereby causing symptoms of poisoning, such as hyperexcitation, ataxia, convulsions, hypersensitivity, choreoathetosis, tremor, and paralysis [8–10]. Pyrethroids exert their toxic effects primarily by altering gating properties of the sodium channel, which is essential for the generation and propagation of action potential in excitable cells [11,12]. The effects of pyrethroids on the neuronal excitability and sodium channels have been described in both insect and mammalian neurons [9,12–16]. However, little is known of the possible effect of pyrethroids on electrophysiologic properties of the adult honeybee brain neurons. We further investigated effects of pyrethroids, such as fluvalinate, deltamethrin and bifenthrin, on neuronal excitability as well as underlying sodium currents. We found that pyrethroids greatly suppressed the neuronal excitability of honeybee and sodium channel of nerve membrane, which might account for the changed egg laying behavior of honeybees exposed to pyrethroids.

2. Materials and methods

2.1. Chemicals

Bifenthrin, (2-methylbiphenyl-3-ylmethyl (Z)-(1RS,3RS)-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate), was obtained from FMC corporation, in the form of 2.5 EC. Deltamethrin, (S)-alpha-cyano-3-phenoxybenzyl (1R,3R)-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropancarboxylate, was obtained from Bayer, in the form of 2.5 EC. Fluvalinate ((RS)-α-cyano-3-phenoxybenzyl-(R)-2-[2-chloro-4-(trifluoromethyl)-anilino]-3-methylbutanoate) (91% purity) was obtained from Mitsubishi Chemicals Corporation.

2.2. Honeybees for behavioral observation

Experiments were performed at LC5 doses of bifenthrin, deltamethrin and fluvalinate to honeybee colonies and repeated in

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doi:10.1016/j.pestbp.2011.02.001

three consecutive years (May 2006, July 2007 and September 2008). The plants around the apiary did not have nectar throughout the experimental period. Colonies that had been treated with a pyrethroid in the previous year were excluded from new experiment and different colonies were used for each year's experiments. Test colonies were headed by sister queens in the same year. The bees were divided into groups of five colonies. The test colonies were numbered and each test group labeled with the study number, and treatment. Colonies were allowed to fly freely. Each treated colony was fed pesticide solution (400 ml per day) at a concentration (LC5: 6.9 mg/L bifenthrin, 21.6 mg/L deltamethrin, 184.5 mg/L fluvalinate) that was derived from the toxicological tests [17]. Each queen of treated colony was directly fed 5 μl of pesticide solution every 5 days. Pesticides were introduced in a liquid mixture of 1:1 sucrose:water. Control colonies were fed a liquid mixture of 1:1 sucrose:water. All of the colonies were fed for 20 days. The stored honey and syrup were taken out every 3 days in order to avoid the effects of pesticides concentrated in the stored honey. Pesticides were not sprayed onto plants around the apiary. During the period of the test, the locomotor activity of workers and egg laying behavior of queens were observed. The percent of one cell one egg per queen per day was counted respectively. After test, the normal rate of the laying-eggs of the honeybee *Apis mellifera ligustica* from bifenthrin, deltamethrin, fluvalinate and control were compared in 2006, 2007 and 2008.

2.3. The model of chronic effects of pyrethroids on honeybees for electrophysiological test

Bee workers (*Apis mellifera ligustica*) were captured from honey and pollen combs in the hive and were mainly foragers. The bees were stored in $9 \times 9 \times 6$-cm cages. All bifenthrin, deltamethrin and fluvalinate solutions were prepared in liquid mixture of sucrose and water (1/1 w/v). Bees were fed bifenthrin, deltamethrin and fluvalinate in sucrose and control bees were fed untreated sucrose. The final concentrations of bifenthrin, deltamethrin and fluvalinate in sucrose solutions were 6.9, 21.6, and 184.5 mg/L respectively. Control bees were fed a liquid mixture of sucrose and water (1/1 w/v). All bees were fed for 5 days and at the end of the fifth day, mortality of treated bees was less than 5%. The cages were placed in the incubator (30 ± 1 °C, 60 ± 10% relative humidity, darkness).

2.4. Honeybee brain neuron preparation

Dissociation and culture of neurons from honeybee brains was made according to the modified protocol from Kreissl and Bicker [18]. Briefly, honeybee brains were removed from the head capsule in a L15 medium (Gibco BRL) supplemented with sucrose and glucose 42.0, 4.0 g/L, respectively. After incubation (10 min) in a calcium-free saline solution (in mM 130 NaCl, 5 KCl, 10 $MgCl_2$, 25 glucose, 180 sucrose, 10 Hepes; pH 6.6), brain tissues were transferred to L15 medium containing papain (100 μl in 2 ml) for 15 min, and then the tissue was rinsed twice with L15 and dissociated by gentle trituration. Cell density was adjusted to 5×10^5/ml and cells were then plated into dishes and allowed to settle and adhere to the substrate for 45 min. Thereafter, the dishes were filled with approximately 2.5 ml of culture medium containing 13% (v/v) heat-inactivated fetal calf serum and 1.3% (v/v) yeast hydrolysate. The dishes were filled with 2.5 ml of L15 and were kept at 29 °C in an incubator at high humidity. After 12–18 h, round cells with a diameter of 18–22 μm were selected for electrophysiological recordings.

2.5. Electrophysiological recordings

Whole-cell giga-ohm seal recording was performed at room temperature by means of an Axonclamp 200B amplifier and pClamp9 software (Axon Instruments). Electrodes were fabricated from borosilicate glass and the impedance of a typical patch pipette was 2–4 MΩ. To record the neuronal excitability, the pipette solution contained (in mM): 115 potassium gluconate, 40 KF, 20 KCl, 3 $MgCl_2$, 1 $CaCl_2$, 2 Mg–ATP, 2 Li–ATP, 11 EGTA, 120 sucrose and 10 Hepes; pH 6.7. For the sodium current recordings, the pipette solution contained (in mM): 120 Cs-fluoride [19], 30 TEA-Cl_2, 2 $MgCl_2$, 1 $CaCl_2$, 2 Mg–ATP, 2 Li–ATP, 11 EGTA, 160 sucrose, adjusted to pH 6.7. For all recordings, the bath solution contained (in mM): 130 NaCl, 6 KCl, 5 $CaCl_2$, 4 $MgCl_2$, 25 glucose, 160 sucrose, 10 Hepes; pH 7.4. For the sodium current recordings, the bath solution contained 0.1 mM $CdCl_2$ to block Ca^{2+} currents.

2.6. Statistical analysis

The selected concentration LC5 values were determined by the probit analysis program of Russell et al. [20]. Statistical analyses were performed using SAS™ (Cary, NC, USA) [21]. The normal rate of the laying-eggs of the honeybee for each year after transformed percent to arc-sin were analyzed separately using one-way analysis of variance (ANOVA) with type of pesticide (bifenthrin, deltamethrin, fluvalinate and control) as the independent variable. Each colony was treated as an experimental unit. When significant differences were found ($p \leqslant 0.05$), multiple comparison procedures were performed by Tukey's honestly significant difference (HSD) tests. One-way ANOVA followed by the Tukey's post hoc test was used to test the differences in the current threshold, sodium peak current or voltages of half inactivation of the sodium currents. The differences in the kinetics of activation of the sodium currents before and after pyrethroid treatments were analyzed using two-way repeated measure ANOVA.

3. Results

3.1. Effect of pyrethroids on egg laying behavior in honeybee queens

We investigated the behavioral changes of laying-eggs in queens treated with fluvalinate, deltamethrin and bifenthrin. In normal circumstances, queens lay eggs in cells orderly, with one egg in one cell. After treatment with bifenthrin, queens laid eggs disorderly and unsystematically, with multiple eggs in one cell (a maximum of eight eggs in one cell), but fluvalinate or deltamethrin treatment did not result in this unusual egg laying behavior. Queens fed bifenthrin had a lower normal rate of laying-eggs compared with the control, deltamethrin and fluvalinate across the three year period ($p < 0.0001$; Table 1).

3.2. Acute effects of pyrethroids on neuronal excitability

Excitability of honeybee brain neurons was evaluated by changes in the current threshold, defined as the minimal current for generation of action potentials. We first investigated the effects of pyrethroids on the current threshold in the acute dissociated honeybee brain neurons. To measure the current threshold before and after exposure to pyrethroids, under the whole cell current clamp configuration, a series of increasing current steps, each of 500 ms duration, were delivered in increments of 10 pA starting from −50 pA to the recorded honeybee brain neurons. Before exposure to the pyrethroids, the neurons responded to a depolarizing stimulus by firing one action potential [22]. After a local, puff application of fluvalinate, deltamethrin and bifenthrin at a concen-