Deterrence of feeding in *Rhodnius prolixus* (Hemiptera: Reduviidae) after treatment of antennae with a nitric oxide donor

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Abstract. The blood-sucking bug *Rhodnius prolixus* is the main vector of Chagas Disease in Colombia, Venezuela and several countries in Central America. Nitric oxide (NO) is a ubiquitous gaseous molecule present in most types of cell and participates in the olfactory pathway of insects. In this work, nitroso-acetyl-cysteine (SNAC), a nitric oxide donor, was topically applied to the antennae of fifth instar nymphs of *R. prolixus*. After SNAC treatment, these insects showed a dose-dependent reluctance to feed when provided with a living pigeon as the food source (ED₅₀ = 5.2 µg/insect). However, there was no reluctance to feed when db-cGMP was applied to the antennae of nymphs. In another experiment, insects that had their antennae treated with SNAC were less attracted than the control group to a CO₂ source. A possible role of NO in the olfactory pathway of *R. prolixus* is discussed.

INTRODUCTION

The blood-sucking bug *Rhodnius prolixus* is the main vector of Chagas Disease in Colombia, Venezuela and some areas of Central America (Schofield, 1994). This insect detects several chemicals that emanate from the host, which elicit an attractive behavioural response (Barrozo & Lazzari, 2004b). Among these substances, are volatile compounds emitted from the human skin (lactic acid, short and long chain fatty acids, aldehydes) and CO₂ present in human breath (Barrozo & Lazzari, 2004a). Carbon dioxide and other volatiles are involved in long-range orientation, while a thermal cue seems to be more important for the close-range orientation (Núñez, 1987). Chemical signals are detected by specific receptors present on the bug's antennae (Flores & Lazzari, 1996).

Nitric oxide (NO), a ubiquitous gaseous molecule present in most types of cell, participates in the olfactory pathway of insects (Bicker, 1998; Davies, 2000). The main function of this membrane-permeating molecule is the activation of soluble guanylate cyclase (GC) activity, leading to the formation of cGMP in target cells (Müller, 1997). Neuro-chemical investigations of NO/cGMP signalling in the nervous system of insects suggest it has critical functions in the olfactory pathway, vision and mechanosensation (Bicker, 2001).

Olfactory receptor cells respond to stimulation from odours by depolarization of the cell membrane. There is an underlying mechanism of transduction, which converts the electrical signal into a chemical one, generally mediated by cyclic nucleotides such as cGMP, cAMP and IP₃ (Breer et al., 1992). The olfactory cyclic nucleotide-gated Ca⁺⁺ channel in rat is composed of two subunits (α and β). The β subunit is activated in vitro by NO-generating compounds (Broillet & Firestein, 1997). This result indicates that NO could also change the Ca⁺⁺ permeability by direct union with the cation channel instead of increasing cyclic nucleotide concentration by activation of GCs.

Studies on a moth show that in the presence of sexual pheromone and NO the concentration of cGMP in olfactory receptor

neurons (ORNs) increases (Strengl et al., 2001). However, in situ hybridization experiments show that NO-dependent soluble GCs are not expressed in pheromone-sensitive ORNs (Broillet & Firestein, 1997; Strengl et al., 2001). These results suggest that the intracellular rise in cGMP concentration is involved in processing pheromone-related information, mediated by an NO-sensitive-mechanism rather than a NO-dependent soluble GC.

Most studies on NO and the sensorial function in insects have used biochemical (Stengl & Zintl, 1996; Redkozubov, 2000; Newland & Yates, 2008) or electrophysiological (Stengl et al., 2001; Waserman & Itagaki, 2003; Dolzer et al., 2008) methods. However, the insect's behavioural response was only studied in a few cases, mainly in the locust (Newland & Yates, 2007; Wenzel et al., 2005). In a previous study, we show that treating *R. prolixus* nymphs with the NO donor S-nitroso-N-acetyl-cysteine (SNAC) produces a decrease in the behavioural response to the classical insect repellent N,N-diethyl-3-methylbenzamide (DEET) (Sfara et al., 2008). A similar result is obtained when the antennae of nymphs are treated with the cGMP analog dibutiryl-cyclic-guanosine-monophosphate (db-cGMP). The aim of the present work was to evaluate the effect of SNAC or db-cGMP treatments of antennae on the feeding behaviour of *R. prolixus*.

MATERIAL AND METHODS

Biological material

R. prolixus is a haematophagous hemipteran that has five nymphal instars. Fifth instar nymphs of this insect, obtained from a strain kept in our laboratory since 1985, were used in all bioassays. Nymphs were starved for 30–50 days after emergence and kept in a temperature-controlled chamber at 28°C, under 12L : 12D photoperiod.

Chemicals

SNAC was used as an NO donor. It was synthesized in our laboratory by acid-catalyzed nitrosation of acetyl-L-cysteine as

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described by Mathews & Kerr (1993). Following this protocol, a 40 mM solution of SNAC in acetone was obtained. Acetone, analytical grade, was purchased from Merck (Darmstadt, Germany) and db-cGMP was purchased from Sigma-Aldrich (Milwaukee, WI, USA).

Feeding behaviour bioassays

The first experiment was designed to evaluate whether SNAC deterred nymphs of *R. Prolixus* from feeding. A 40 mM solution of SNAC in acetone was serially diluted to obtain a total of five concentrations. Each concentration was assigned to an insect group. Solutions were topically applied to the antennae of insects using a micro-syringe provided with a dispenser (Hamilton, Reno, NV). Each insect received 1 µl per antenna, and the following concentrations of SNAC were used: 0.9375, 1.875, 3.75, 7.5 and 15 µg/insect. The control group was treated with the same volume of pure acetone.

Groups of five nymphs were taken from the rearing cage 24 h before the experiment and each marked with acrylic yellow paint on the dorsal side of the thorax for identification. Immediately before the experiment, the weight of each insect was recorded and then the SNAC solutions were applied. Immediately, a food source (pigeon) was offered to the insects for 15 min. Finally, the insects were individually weighed and the number of fed and unfed nymphs was determined by the differences in weight before and after the exposure to the pigeon. The percentage of insects that fed was determined for every concentration of SNAC applied. Six independent replicates were performed.

To investigate the possible role of cGMP in deterring feeding, we performed a second experiment as described above, but the antennae of each insect were treated with acetone solutions of db-cGMP. Two concentrations of this compound were applied: 1 or 2 µg/insect. These concentrations were chosen based on a previous study in our laboratory, which indicates a dose-dependent decrease in the behavioural response to DEET when topically applied to the antennae of fifth instar nymphs of *R. prolixus* (Sfara et al., 2008).

Response to CO₂

In the third experiment, the arena consisted of a plastic circular container 20 cm in diameter with its floor covered by a sheet of filter paper. In the center of the arena, a source of CO₂ was placed. The CO₂ was released by the reaction of NaHCO₃ with H₂SO₄ 50% w/w. At the start of the experiment, 30 mg of NaHCO₃ was weighed in a plastic container with a diameter of 2 cm and placed in the center of the arena, where the insects had already been present for 5 min. Then, 50 µl of H₂SO₄ were added to the NaHCO₃, and CO₂ was immediately generated. Two areas were defined: a central area around the CO₂ source and a peripheral area.

A group of ten nymphs was tested per dose. Two doses of SNAC were applied: 1.5 or 15 µg/insect. One µl per antenna of each solution was applied to each insect. The same volume of pure acetone was applied to the controls. In a preliminary assay, the number of visits to the central area was 0 in the absence of a source of CO₂, so we did not include this treatment in this experiment.

Movement of the insects was recorded with a video camera and observed on the screen of a monitor. The number of insects that visited the central area was recorded for 15 min. Eight independent replicates were performed.

Statistical analysis

An ED50 value for the feeding deterrent effect of SNAC (the effective dose that deterred feeding in 50% of the treated insects) was calculated using the Polo Plus 2.0 program (LeOra

Fig. 1. Percentage of fed insects after treatment with different doses of SNAC topically applied to the antennae of *R. prolixus* nymphs. ED50 – Effective Dose 50%. Abscissa is scaled logarithmically and ordinate using a probit scale.

Software, 1987). Percentages of unfed insects were determined and transformed to arcsine square-root values, prior to the analysis of variance (one-way ANOVA).

In the experiment on the attraction to CO₂, the mean number of visits to the central area was calculated and compared statistically using one-way ANOVA followed by Tukey's test for post hoc comparisons.

RESULTS

When the antennae of insects were topically treated with SNAC, a dose-dependent deterrent effect on feeding was recorded (Fig. 1) and the ED50 value was 5.2 µg/insect (95% CI: 2.8–23.2) based on the results of six replicates. It was observed that some of the unfed insects extended their proboscis but did not feed. This behaviour was recorded for 28% of the insects that did not feed but it was not possible to establish any correlation between this effect and the dose of SNAC applied. Topically treating the antennae of nymphs with db-cGMP did not significantly affect their feeding behaviour ($P > 0.05$, one-way ANOVA; six replicates) (Fig. 2).

The results obtained when antennae were treated with SNAC and insects were exposed to CO₂ are shown in Fig. 3. A signifi-

Fig. 2. Percentage of unfed insects after treatment with different doses of db-cGMP topically applied to the antennae of *R. prolixus* nymphs. Vertical lines are SE. Differences among treatments are not significant ($P > 0.05$, one-way ANOVA).

Fig. 3. Mean number of visits of *R. prolixus* nymphs to the central area of an experimental arena where there is a source of CO₂. Vertical lines are SE. Different letters indicate significant differences among treatments ($P < 0.05$, one-way ANOVA; Tukey's test for post-hoc comparisons).

cantly lower number of visits to the central area were recorded for the group of nymphs treated with 15 µg/insect of SNAC ($P < 0.05$, one-way ANOVA and Tukey's test for post hoc comparisons; eight replicates).

DISCUSSION

There are some reports that relate the NO/cGMP pathway with mechanisms of sensory adaptation in insects. Several studies indicate that cGMP-dependent signal transduction cascades underlie the adaptation of the olfactory system of moths during minute-long or exposure to strong odours. Redkozubov (2000) reports a possible role of guanylate cyclase and cGMP in the sensory adaptation of the olfactory receptor neurons (ORNs) of the silk moth *Bombyx mori*. This author observed that the responses of the ORNs to high concentrations of pheromone decrease when insects are treated with the cyclic nucleotide analogue, db-cGMP. An elevated concentration of cGMP reduces the pheromone induced IP₃ response, suggesting that cGMP could be involved in the adaptation of the antennal receptors cells of *Heliothis virescens* (Boekhoff et al., 1993). The regulation of the generation of cGMP through the activation of guanylate cyclase, responsible for cGMP synthesis, is involved in the adaptation of the olfactory cells of *Antheraea polyphemus* and *Bombix mori* (Ziegelberger et al., 1990). These results are also supported by the finding of different types of guanylate cyclase in *M. sexta* (Simpson et al., 1999; Nighorn et al., 2001; Stengl et al., 2001; Morton & Nighorn, 2003).

There is evidence that NO increases cGMP concentration (Stengl et al., 2001) and activates ion channels in the ORNs of *M. sexta* (Zufall & Hatt, 1991; Dolzer et al., 2008). Stengl & Zintl (1996) found NADPH diaphorase activity (indicative of NO synthase activity) in ORNs and olfactory supporting cells of *M. sexta*.

Electrophysiological studies show that nucleotide-activated currents in cultured ORNs of *M. sexta* are directly activated by cAMP and cGMP (Krannich & Stengl, 2008); and that a cGMP analog adapted the action potential response of pheromone-sensitive sensilla trichoidea of *M. sexta* in a daytime-dependent manner (Flecke et al., 2006).

In a previous study, we demonstrate that the behavioural response of *R. prolixus* to DEET decreases after prolonged exposure to this repellent, suggesting a possible adaptation phenomenon (Sfara et al., 2008). We also observed a decrease in

the response to DEET after treating *R. prolixus* antennae with the NO donor SNAC or db-cGMP, which might indicate that the NO/cGMP pathway is involved in adaptation. In the present study, we found that the treatment of *R. prolixus* antennae with SNAC resulted in a dose-dependent deterrent effect on feeding. However, feeding was not deterred when insects were treated with db-cGMP. This indicates that feeding in *R. prolixus* can be deterred by a NO-sensitive mechanism but not by NO-dependent guanylate cyclase. Decreases in sensitivity of the sensory system may be due to changes in the permeability of ion channels, mainly by increases in the intracellular Ca⁺⁺ concentration. Increase in intracellular Ca⁺⁺ is usually caused by the activation of CNG Ca⁺⁺ channels (Stengl, 2010). Broillet & Firestein (1997) describe a Ca⁺⁺ channel directly activated by NO in rats. This channel has a β subunit that can be activated by union with NO.

We studied the orientation of SNAC-treated triatomines towards CO₂, a chemical clue they use to locate hosts (Núñez, 1987). A significantly lower number of insects were attracted to the CO₂ source when the higher dose of SNAC was applied. These results accord with the behaviour recorded during the feeding experiments: when placed on a living pigeon, treated insects did not identify the food source and walked around the walls of the container throughout the whole period of the experiment.

The decrease in the response to the DEET repellent (Sfara et al., 2008) and the reluctance to feed as well as the decrease in the response to CO₂ of insects treated with SNAC reported here, are consistent with previous studies that suggest that the NO/cGMP is the signalling system of the olfactory adaptation process.

In summary, this study indicates that *R. prolixus* is deterred from feeding and its attraction to CO₂ is reduced when the NO donor SNAC is topically applied to its antennae. Further studies are needed for a better understanding of the role of NO and the transduction mechanisms involved in chemoreception during feeding in *R. prolixus*.

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