Alarm Pheromone Induces Stress Analgesia via an Opioid System in the Honeybee

Josué Núñez, Lourdes Almeida, Norberto Balderrama, and Martin Giurfa

*Laboratory of Insect Physiology, Department of Biological Sciences, FCEyN, Universidad de Buenos Aires, 428 Buenos Aires, Argentina; †Instituto Zoología Agrícola, Facultad Agronomía, Universidad Central de Venezuela, Aptdo. Postal 4579, Maracay 2101, Aragua, Venezuela; ‡Institut für Neurobiologie, Freie Universität Berlin, Königin-Luise-Str. 28/30, 14195 Berlin, Germany

Received 3 March 1997; Accepted 29 July 1997

Núñez, J. A., L. Almeida, N. Balderrama, and M. Giurfa. Alarm pheromone induces stress analgesia via an opioid system in the honeybee. PHYSIOL BEHAV 63(1) 75–80, 1998.—Changes of the stinging response threshold of Apis mellifera scutellata were measured on foragers fixed on a holder and stimulated with an electric shock as a noxious stimulus. The threshold of responsiveness to the noxious stimulus increased when bees were previously stimulated with isopentyl acetate, which is a main component of the alarm pheromone of the sting chamber. This effect is antagonised by previous injection of naltrexone-hydrochloride (Endo Laboratories Inc.). Results suggest that in the honeybee an endogenous opioid system activated by isopentyl acetate is responsible for modulation of perception for nociceptive stimuli. The resulting stress-induced analgesia in the defender bee would reduce its probability of withdrawal thus increasing its efficiency against enemies. © 1998 Elsevier Science Inc.

Honeybees Opioid system Alarm pheromone Stress-induced analgesia Stinging response

Mechanisms of “stress-induced analgesia” in relation to defensive behavior have been postulated in several animals (5). Such mechanisms would increase the threshold of responsiveness to external stimuli that elicit innate defensive responses through activation of endogenous opioid and non-opioid mediated forms of stress-induced analgesia (17).

Although the analgesic properties of opiates in vertebrates are well established (14), less is known about the presence and role of such substances in invertebrates. Studies on the role of opioids in relation to nociception and stress-induced analgesia have been conducted in molluscs (12,9), in insects (16,18,6,2,11,10), and crustaceans (13). Honeybees Apis mellifera seem to be particularly well suited for studying this question. As members of a colony, they exhibit defensive responses that can even lead to death. When disturbed at the hive entrance, honeybee workers may exhibit “alarm fanning” and release an alarm pheromone by which they recruit hive-mates for defense against intruders (“Stachel” or “Poison-warning signal”; see [8]). One of the major components of the alarm fanning display is the “stinging response” (SR), i.e., a full opening of the sting chamber and complete protraction of the sting (16). This reaction may be reproduced under controlled conditions by means of an electrical stimulation (2 s with 8-volt 100 Hz, 1 ms biphasic pulses) applied on isolated foragers fixed on a holder (16). In this experimental paradigm, the threshold for the SR increased (i.e., the SR is inhibited) when workers were injected with morphine, and the observed effect was antagonised by application of naltrexone, (Endo Laboratories, Inc.). These results demonstrated the occurrence of opiate receptors in the honeybee and suggested for the first time that an endogenous opioid system might be involved in the modulation of the stinging response in the honeybee (16,2).

In this study, we ask whether isopentyl acetate (IPA), a major component of the sting alarm pheromone (7), activates per se the endogenous opioid system, and therefore modulating the stinging response. Because the alarm pheromone is released in a defensive context and signals a potential enemy, it might activate the opioid system to produce stress-induced analgesia. If this is the case, a nociceptive stimulus eliciting a stinging response would not be as successful if bees are previously exposed to IPA. Contrarily, if bees are exposed to naltrexone and IPA, the same nociceptive stimulus would produce a more consistent stinging response due to the antagonising effects of Naltrexone on the opioid system activated by IPA.

Materials and Methods

Experiments were conducted from October to December 1989, at the Instituto de Zoología Agrícola, Facultad de Agronomía, UCV, Maracay (10° North; 67° West; 446 m above sea level), Venezuela. Temperature normally fluctuated between 25° and 30°C.

Subjects. Honeybees of the africanized strain Apis mellifera scutellata Lepeletier from hives 20 m distant from the laboratory...
Ten of these bees were injected with 2 separate vials, and inactivated by putting the vial in ice for ca. 3–5 min (Fig. 2b). Those parts of the bee that had been fitted into the plates and those parts of the bee's peduncle and the bee's neck were tightly fitted into the notch N1 and the bee's peduncle was immobilized, and contact with the middle or hind legs was avoided. Plates E1 and E2 were connected to the output of the stimulator (100 Hz, 1 ms biphasic pulses). Notches N1 and N2 were smoothed with an EEG-cream (Spectra 360 Electrode Gel, Parker Laboratories Inc.) in order to obtain good contact between the plates and the bee. After syrup intake, 20 bees were captured (Fig. 2a), each in a separate flask, together with a vial containing a filter paper with 25 μL of paraffin oil (paraffin oil group). The other two were confined for 30 min, each in a separate flask, together with a vial containing a filter paper with 25, 50, or 100 μL of IPA in paraffin oil in a proportion of 1:9 volume/volume [IPA groups (Fig. 2e)]. Therefore, the following treatment groups were obtained (c/c = control group):

- H2O/paraffin oil group (c/c): 5 bees
- H2O/IPA (either 25, 50, or 100 μL IPA) (c/IPA): 5 bees
- NX/paraffin oil groups (either 1, 2, or 4 nM of Naloxone) (NX/c): 5 bees
- NX(2 nM)/IPA groups (either 25, 50, or 100 μL IPA) (NX/IPA): 5 bees

The same procedure was replicated 8 times with a total number of 40 bees per group. In total, 12 groups were used, involving 480 bees (i.e., 3 c/c; 1 c/IPA 25, 1c/IPA 50, 1 c/IPA 100; 1 NX(1nM)/c; 1 NX(2nM)/c, 1 NX(4nM)/c; 1 Nx/IPA 25, 1 Nx/IPA 50, and 1 Nx/IPA 100). For antagonizing the effects of IPA, only the dose of 2nM naloxone was used (NX/IPA groups) because it had already proved to be adequate to counteract the inhibitory effect of morphine on the stinging response (16).

One hour after capture (30 min recuperation time + 30 min confinement time) bees from the different groups were again inactivated at 0°C (Fig. 2f) and individually mounted on the holder (Fig. 2g). After 15 min of recuperation on the holder (controls with 30 min recuperation showed no difference in responsiveness) and under a dissection microscope, bees were electrically stimulated (100 Hz, 1 ms biphasic pulses) for 2 s with 2, 4, 8, and 16 V and with a 1 min interval between stimuli. The voltage range used proved to be an effective disturbance to interrupt drinking in foragers conditioned to collect at an artificial food source, but it was not enough to inhibit further regular visits to the same source (15). The voltage scanning used in this study allowed us to find a voltage for maximal responsiveness.

For a given voltage, SR was scored 1 when the sting was totally exposed by opening the sting chamber during the 2 s of the stimulation period (Fig. 1b; SR = 1); shorter SRs or partial ones were scored 0.

**Results and statistical analysis.** The sensitivity of bees to the noxious stimulus was quantified by ranking the bee's responses according to a SR-Index.

\[
\text{SR index} = \frac{\text{[number of bees with SR/total number of bees tested]}}{\text{SE}}
\]

Results were expressed as the mean value of SR indexes ± SE. A comparison between treatment groups and controls was carried out by means of two-way factor repeated measurements ANOVA (factor 1: voltage; factor 2: treatment group). Comparison between means was performed by means of a Newmann–Keuls test (19).

**Results**

The dependence of the SR-Index on the intensity of the electrical stimulation is depicted in Fig. 3a for the c/c and the c/IPA groups (c/IPA 25 μL, c/IPA 50 μL, and c/IPA 100 μL). The responsiveness to the noicceptive, electrical stimulus was greater in the c/c group than in the groups treated with IPA. There was a...
significant variation according to the treatment group ($F = 12.88$; df: 3, 60; $p < 0.001$) and the voltage employed ($F = 19.88$; df: 3, 180; $p < 0.001$). In the same way, the interaction voltage*treatment was also significant ($F = 6.44$; df: 9, 180; $p < 0.001$). With a 2V-stimulation, no significant differences between groups were detected (Newmann–Keuls test, $a = 0.05$). With increasing voltage, significant differences between the c/c and the c/IPA curves appear, particularly from 8 V onward. These differences are enhanced for the c/IPA 100 μL curve that did not vary with the voltage applied.

Fig. 3b presents the SR index obtained for bees previously injected with 2 nmol of naloxone hydrochloride and subsequently exposed to IPA (Nx/IPA-25, 50, and 100 μL groups). The Nx/IPA-groups were not significantly different from the c/c group ($F = 0.83$; df: 3, 44; ns). All curves showed the same significant pattern of variation with voltage ($F = 31.72$; df: 3, 132; $p < 0.001$), thus, the interaction was not significant ($F = 0.87$; df: 9, 132; ns). In other words, the modulating effects of IPA on the SR were fully antagonized by the previous injection of naloxone (compare Figs. 3a and 3b).
interaction not significant (F on an endogenous opioid system. Of IPA on the SR (Fig. 3a) was actually due to the action of IPA water-injected bees. This demonstrates that the modulating effect, words, bees injected with 1 to 4 nmol of naloxone behaved like

FIG. 3. a) SR Index (ordinate) in dependence on stimulus voltage (abscissa) for: control, c/c and IPA treated c/25 μL; c/50 μL... 2 nmol naloxone hydrochloride injected, and IPA treated groups (Nx/IPA 25 μL; Nx/IPA 50 μL, and Nx/IPA 100 μL). Means ± SE. Points sharing the same letter do not differ at the 0.05 level, after a Newmann–Keuls test modified for ANOVA for repeated measurements. b) SR-Index (ordinate) in dependence on stimulus voltage (abscissa) for: control, c/c and 2 nmol naloxone hydrochloride injected, and IPA treated groups (Nx/IPA 25 μL; Nx/IPA 50 μL, and Nx/IPA 100 μL). Means ± SE. Points sharing the same letter do not differ at the 0.05 level, after a Newmann–Keuls test modified for ANOVA for repeated measurements.

The SR index was assessed in the groups injected only with naloxone (1, 2, or 4 nM) to test whether the previously observed antagonizing effects resulted from the action of naloxone on the endogenous opioid system, activated by IPA, or from the naloxone injection itself. Fig. 4 shows that no significant differences between the c/c and the Nx/c groups exist (F = 0.72; df: 3, 44; ns). All the curves showed the same significant pattern of variation with voltage (F = 47.82; df: 3, 132; p < 0.001), thus, the interaction not significant (F = 1.67; df: 9, 132; ns). In other words, bees injected with 1 to 4 nmol of naloxone behaved like water-injected bees. This demonstrates that the modulating effect of IPA on the SR (Fig. 3a) was actually due to the action of IPA on an endogenous opioid system.

DISCUSSION

The present study shows that IPA, a major component of the sting alarm pheromone (7), activates the endogenous opioid system of honeybees, therefore changing the threshold of responsiveness to a noxious stimulus. Although we did not measure nociceptive sensitivity directly, changes in it could be inferred from changes in the defensive response that we measured.

Bees treated with IPA and subjected to a noxious stimulus (electrical stimulation) evince a lower SR index than the control bees subjected to the same stimulation. In other words, the responsiveness to a noxious stimulus eliciting a stinging response is lower if bees have been previously exposed to IPA. This change of SR-threshold was dependent on the voltage applied and the quantity of IPA used (Fig. 3a). The differences between control (c/c) and IPA bees are maximal for the c/IPA 100 μL curve. If bees are previously injected with naloxone, the SR index does not change in relation to control bees (c/c; (Fig. 3b)). That is, naloxone antagonises the effect of IPA on the SR index, although injection of Naloxone alone has no effect on the SR index as related to that of control bees (c/c; (Fig. 4)).

The findings of the present study can be understood if one assumes that: 1) the responsiveness to actual damage decreases if bees are exposed to a component of the sting alarm pheromone (i.e., SR threshold increases), and 2) the decrease in responsiveness is mediated by an opioid mechanism.

It is worth mentioning that our results are consistent with the finding that the sensitivity/responsiveness of rats to a painful injection of formalin is reduced if the animal is tested in the presence of the odors of stressed conspecifics (4). This analgesia was reversed by naltrexone, suggesting that it has an opioid component. In the same way, studies using the forced-swim test in rats have shown that stressed animals immersed in water swim vigorously for a few minutes and release a low volatile, alarm pheromone that acts as an analgesic for conspecifics subsequently immersed in the same water (1). Together with our findings, these results suggest that stress-induced analgesia, through an alarm pheromone activating the opioid system, might be a general defensive response.

Such an attenuating effect of IPA on the SR seems to be paradoxical because the simultaneous stimulation with sting alarm pheromone and a noxious agent should be expected to induce an increase in the stinging reactivity. However, the result appears no longer paradoxical when the SR is thought of as involving two separate functions: the individual response to actual damage and the social signalling of potential damage to the hive.

After a mild perturbation at the hive entrance, an individual bee becomes alert and assumes a typical attitude with short periods of immobilization (3). With increasing perturbation, it responds with release of alarm pheromone, active locomotion, telescoping abdominal movements, sting response, and “fanning.” The alarm pheromone decreases the responsiveness to a noxious stimulus through the activation of an opioid analgesia. Thus, the individual defensive efficiency would increase because the probability of withdrawal when facing an enemy would be reduced.

The adaptive value of this system for the collective defensive efficiency of the hive is evident because IPA would have a double function: an informational one, as pheromone informing about the presence of potential danger and/or enemy, and an analgesic one, acting on the endogenous opioid system of other hive-mates. This idea is in keeping with a tenet of Fanselow’s “Perceptual-defensive recuperative model” (5). One component of such a model is the labelled “defensive motivational system,” a neurophysiological network that selects appropriate defensive responses to a variety of environmental danger signals. As in other animals, nociceptive stimuli activate the defensive system of the bee and, as a consequence, the behavioral repertoire becomes dominated by species-specific defensive reactions. The defensive attack of the enemy ends mostly with the death of the guardian bee, and the
source of perturbation remains marked with alarm scents associated with the sting chamber.

It may be argued that the confinement of the bees, and not IPA, constitutes the inescapable stressor activating the endogenous opioid system. However, control bees were also confined in a flask (without IPA) and did not show such an activation. This means that the inescapable stressor was the exposition to IPA and not the confinement in a flask. This points out an essential difference between the natural defensive system of the bee evoked by stimulation with IPA and the experimental models mentioned in the literature that use inescapable stressors as aversive stimuli. In a natural context, the conflictive situation between attack and escape in bees defending the colony would be a result of their social binding. It could be assumed that the conflict between the drive to escape and the binding to the society constitutes an inescapable stressor. In a natural context, the conflictive situation between attack and escape in bees defending the colony would be a result of their social binding. It could be assumed that the conflict between the drive to escape and the binding to the society constitutes an inescapable stressor. In this situation, a stress-induced analgesia occurs as a result of the two mutually exclusive responses.

In a previous work (2), it was found that annual fluctuations in the SR appear in both European, A. m. ligustica, and Africanized, A. m. scutellata, bees. Interestingly, despite of the “aggressive” behavior of the Africanized line, its SR was generally lower than that of the European one. This represented, in some way, a surprising result because it was expected that responsiveness would be higher in an “aggressive” strain than in a “tame” one. Now it is possible to interpret these results. The lower SR in Africanized bees would not indicate a lower aggressiveness but a better analgesic modulation through an endogenous opioid system in response to a noceptive stimulus. In other words, the lower SR Index of Africanized bees faced with a noceptive stimulus constitutes a more efficient defensive strategy through reduction of the withdrawal probability.

ACKNOWLEDGMENTS

The authors thank W. Farina, C. Lazzari, H. Maldonado, R. Menzel, F. Roces, and three anonymous referees for comments and corrections on earlier versions of the manuscript.

This work was supported by Grants PID 3-367 200/92 from Consejo de Investigaciones Cientificas y Tecnicas (CONICET), Argentina, and the Alexander von Humboldt-Stiftung, Germany, and the Alexander von Humboldt-Stiftung, Germany, to J. A. N.; B-1417-1 from the International Foundation for Science (IFS), Sweden, to L. A.; 01.38.3313/94 from the Consejo de Desarrollo Cientifico y Humanı́stico (CDCH), UCV, Venezuela, to N. B., and B-1704-2F from the International Foundation for Science (IFS), Sweden, to M. G.

REFERENCES