Lack of Responsiveness of Malpighian Tubules to the AVP-like Insect Diuretic Hormone on Migratory Locusts Infected with the Protozoan *Malameba locustae*

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In 1989–1990, our colony of *Locusta migratoria* was seriously infected by a protozoan *Malameba locustae*. This infection was then rooted out thanks to an appropriate sulfamide treatment. Since this parasite is known to infest chiefly Malpighian tubules, we studied the ability of these organs to respond to their main regulatory factor: the arginine–vasopressin-like insect diuretic hormone (AVP-like IDH). Results demonstrated that the ability of infected Malpighian tubules to respond to the hormonal stimulus is dramatically decreased, likely because of damage at the receptor level. The rest of the tissue is less affected and it remains able to play its excretory role as soon as the cellular working is directly stimulated (when using second messengers for example). The ability of the infected locusts to synthesize AVP-like IDH is not affected either since noninfected locusts are stimulated with hormonal extracts of infected animals. This insensitiveness of the infected locusts to their own diuretic hormone is transient and stops when the infection is over. © 1991 Academic Press, Inc.

KEY WORDS: *Malameba locustae; Locusta migratoria;* diuresis; AVP-like insect diuretic hormone; Malpighian tubules.

INTRODUCTION

Malameba (or *Malpighamoeba*) *locustae* is an amoebia protozoan known to infect Acradidae (King and Taylor, 1936). This organism has been found in numerous laboratory grasshoppers where its presence can be endemic.

Although the worst consequence of the infection is a steady decline in fecundity leading to loss of the colony, it will have, in most of the cases, more pernicious consequences, especially when it is not discovered. It is likely that infection seriously disturbs the host physiology since it affects several biological functions: fat body growing, protein metabolism, oocyte development (Papillon and Cassier, 1978) leading to an important weight loss (see below). On that account infested animals are unfit for performing experimental studies.

Since the protozoan strikes chiefly the Malpighian tubules, causing major structural alterations (Martoja, 1969; Harry and Finlayson, 1976), diuresis is possibly the first physiological factor to be affected.

We took advantage of a severe infection of our colony to study the consequences of infection on the hormonal control of the water balance of the migratory locust, *Locusta migratoria*. Its excretory process takes place via the Malpighian tubules which produce a primary urine and via the rectum where selective reabsorption occurs. A diuretic hormone, the arginine–vasopressin-like insect diuretic hormone (AVP-like IDH), regulates the activity of the Malpighian tubules (Proux et al., 1982, 1988) and an antidiuretic hormone, the neuroparsin, regulates that of the rectum (Fournier and Girardie, 1988).

The responsiveness of Malpighian tubules to the AVP-like IDH was tested during the infection and compared to that of Malpighian tubules of locust of the same colony after infection, as well as to that of Malpighian tubules of locusts from a noninfected colony. We tried to determine the

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cellular structure responsible for the lack of response by using hormonal extracts, a cyclic AMP analogue, and substances acting on the metabolism of this second messenger.

MATERIAL AND METHODS

Experimental Insects

We used 30-day-old male migratory locusts (*Locusta migratoria*) of colonies maintained at 28–30°C and 50% RH. Locusts were fed fresh wheat, synthetic medium, and powdered milk. They came from our colony, during and after the infection, and from a noninfected colony (Pr. P. Cassier, University of Paris-VI., France).

Treatment of the Infected Colony

Fresh wheat was sprayed with a mixture of three sulfamides prepared as follows: 2 g/liter Fumidil B, 250 mg/liter Clamoxyl, and 25 g/liter Thipyrimeth. Thipyrimeth was prepared as a stock solution from 6 g of sulfamethazine + 8 g of sulfapyridine sodium + 12 g of sulfathiozole sodium per 100 ml of tap water.

Feces of infected animals were carefully discarded and the sand used in the laying-boxes was sterilized regularly.

Selection of Malpighian Tubules

Malpighian tubules and rectum were dissected from infected and noninfected animals, fixed in Bouin–Hollande fixative without acetic acid and embedded in paraffin. Transversal sections (7 μm) were rehydrated and stained with Mason's hemalun and 1% eosin.

When Malpighian tubules were dissected out to be assayed, they were discarded as a whole if only one of them exhibited visible parasites (white accumulation of trophozoites or black cysts). When trophozoites or cysts were not visible, some Malpighian tubules were used to make a smear for microscopic examination. Only locusts completely devoid of parasites were considered noninfected. This control was systematically performed on every Malpighian tubules set used in this study.

Some extractions and in vitro studies were performed in saline developed by Maddrell and Klunsuwan (1973).

Extraction of Tissues and Preliminary Purifications

Thoracic ganglia containing AVP-like IDH (Proux et al., 1980) were extracted in appropriate medium (see below) and homogenized by ultrasonic probe (Ultrasons 150 T S, Annemasse S.A., France) for 30 sec. Each homogenate was then centrifuged for 20 min at 4°C and 10,000*g*. The pellet was reextracted and centrifuged as before and the supernatants were combined. When isolated Malpighian tubules were stimulated with their own AVP-like IDH (thoracic ganglia and Malpighian tubules coming from the same batch of locusts) the extracts were prepared in saline and simply stored at 4°C until the time of the assay. When extracts were prepared as stock solutions or were intended for subsequent prepurification, they were made in 0.2 M acetic acid, dried using a Speed Vac concentrator (Savant Instruments, Farmingdale, New York), and then stored at −20°C or prepurified by batch absorption and elution from disposable reversed-phase cartridges (Sep-Pak C_{18}, Waters Associates, Milford, Massachusetts). Each cartridge was prepared as described (Schooley et al., 1987) and loaded with the extract of ganglia. The cartridge was washed with 1% trifluoroacetic acid (TFA) and then eluted with 30% 1-propanol–0.1% TFA. The propanol–TFA fraction, containing the AVP-like IDH, was dried and saved for bioassay.

More elaborate purification was not necessary since we demonstrated previously that all the diuretic activity of the thoracic ganglia is due to the AVP-like IDH (Proux et al., 1982).

In Vitro Studies

Malpighian tubules bioassay. We used the assay we previously developed (Proux

et al., 1988). Briefly Malpighian tubules were dissected and placed in a laboratory-made device where the excretion of the isolated Malpighian tubules was monitored every 15 min for two periods of 60 min (equilibration then stimulation) or three periods of 45 min (equilibration then two stimulation periods). The excretion of each quarter of the stimulation period(s) was compared to that of the last quarter of the equilibration period and the difference expressed as a percentage. Stimulations were performed with tissue extracts (two-thoracic ganglion equivalent), 10^{-5} and 4.10^{-5} M 8-(4-chlorophenylthio)-adenosine 3',5'-cyclic AMP (CPT cyclic AMP) or a mixture of 10^{-4} M forskolin plus 2.10^{-4} M 3-isobuthyl-1-methylxanthine (IBMX).

Malpighian tubule incubation. Malpighian tubules were incubated in 400 µl of the saline containing 10^{-4} M forskolin and 2.10^{-4} M IBMX. After a 2-, 15-, or 45-min incubation at 25°C the Malpighian tubules were taken from the saline and cyclic AMP was extracted and measured with appropriate radioimmunoassay (RIA).

RIA of Cyclic AMP

Cyclic AMP was extracted (1 M perchloric acid) from Malpighian tubules and measured according to the RIA developed by Cailla et al. (1973) and modified by Volker et al. (1985). Results were expressed as nanomoles of cyclic AMP per milligram of protein. To quantify the proteins, we used the procedure of Bradford (1976).

Chemicals

CPT cyclic AMP, forskolin, and IBMX were from Sigma (St. Louis, Missouri). They were prepared as a stock solution in dimethyl sulfoxide and diluted with saline prior to use (final concentration 2%). ^{125}I-labeled cyclic AMP was custom made (Volker et al., 1985). Antibody against cyclic AMP, raised as described (Cailla et al., 1973), was generously provided by Dr. Cailla. Fumidil B was from Thersa-Prolivalt

(France), Clamoxyl from Beecham Lab. (France), and Thipyrimeth from Coopérative Pharmaceutique Francaise (France).

Statistical Treatment of Data

Standard deviations are shown in graphs. Comparisons were performed by means of variance analysis.

RESULTS

Histological Study

An histological study of both Malpighian tubules and rectum of infected and noninfected locusts was carried out (Fig. 1). Results are absolutely identical to those previously reported (Martoja, 1969; Harry and Finlayson, 1976). The Malpighian tubules structure is heavily affected. Trophozoites invade the lumen of the tubule and damage the brush border of the tubule cells (Fig. 1A). Some of the trophozoites are encysted and the tubules become blackened. The multiplication of cysts cause a swelling and, in some cases, a rupture of the tubule wall. In contrast, no trophozoite has been observed at the rectum level where the structure is unchanged (Fig. 1C). This alteration of the Malpighian tubules causes certainly deep disfunctioning in the host physiology since the body weight is significantly lower ($P < 0.01$) in infected locusts (1190 ± 66 mg) than in noninfected locusts (1520 ± 65 mg).

Lack of Sensitivity to the AVP-like IDH

Infested Malpighian tubules were incubated with extracts of thoracic ganglia subjected (Fig. 2B) or not (Fig. 2A) to a subsequent Sep-Pak purification. Then the urine excretion rates were compared to those of controls bathed with saline only. Urine excretion is slightly increased (Fig. 2A) or remains equivalent to that of controls, i.e., is steadily decreasing (Fig. 2B). Differences with controls were never significant.

This desensitizing can originate from different causes: the alteration of the Malpighian tubules due to the parasite but also
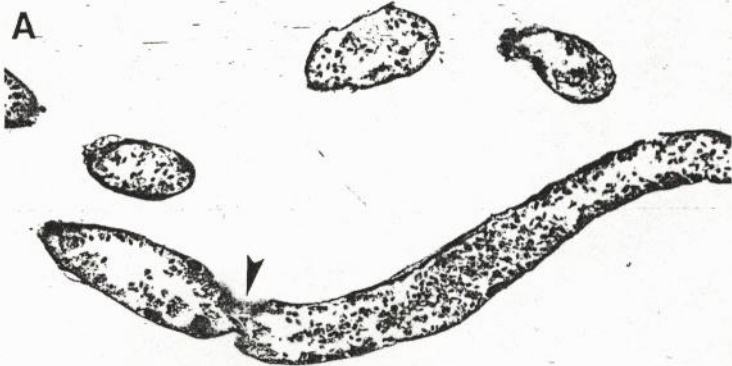
some experimental deficiency or a lack of hormone in the tested extracts. To rule out the two latter hypothesis, several experiments were carried out. AVP-like IDH was extracted from both infected and noninfected locusts and then tested on infected or noninfected Malpighian tubules (coming from Pr. Cassier's colony) (Figs. 3A and 3B). Noninfected Malpighian tubules are always stimulated whatever the origin of the extracts. On the other hand infected Malpighian tubules are always unaffected, or very weakly and slowly affected by the diuretic hormone. The observed desensitizing is due to the infected Malpighian tubules themselves which are no longer able to respond to the hormonal diuretic message possibly because of cellular damage. To determine the cellular compartment involved (receptors, membrane, or cytosol), we used a cyclic AMP analogue, an activator and an inhibitor of the cyclic AMP metabolism, since we demonstrated previously that cyclic AMP is the second messenger of the AVP-like IDH (Proux and Hérault, 1988).

Ability of the Infected Malpighian Tubules to Be Stimulated by a Cyclic AMP Analogue

Infested Malpighian tubules were bathed with CPT cyclic AMP, a cyclic AMP analogue known to easily enter the cells and therefore efficient at low concentration. CPT cyclic AMP triggers a very positive response since the excretion rate is increased by 70% 15 to 30 min after an addition of a 10^{-5} M analogue while it is decreased by almost 10% on controls (Fig. 4).

This stimulation occurs on Malpighian tubules refractory to the diuretic hormone extracts as demonstrated in Figure 5. Malpighian tubules unaffected by diuretic extracts give positive responses with CPT cyclic AMP while Malpighian tubules posi-

tively stimulated by CPT cyclic AMP are no longer responsive when diuretic extracts are applied.

Effect of Forskolin and IBMX on Excretion and Cyclic AMP Level of Infected Malpighian Tubules

A mixture of 10^{-4} M forskolin and 2.10^{-4} M IBMX, by stimulating the adenylate cyclase system and inhibiting phosphodiesterase activity, stimulates both the excretory activity and the cyclic AMP concentration of noninfected Malpighian tubules (Proux and Hérault, 1988). This mixture was tested here on infected Malpighian tubules.

Urine excretion increased significantly (Fig. 6) but this increase is far from being as great as those obtained previously in noninfected tubules (Proux and Hérault, 1988) or obtained above with diuretic extracts or CPT cyclic AMP, and is followed by a period of desensitizing to CPT cyclic AMP. Indeed Malpighian tubules previously stimulated with forskolin plus IBMX are no longer sensitive to CPT cyclic AMP, contrary to those previously bathed in saline. Curiously, this medium-level increase in urine excretion occurs together with a huge increase in cyclic AMP cellular concentration, since forskolin plus IBMX stimulates a 50-fold increase of the cyclic AMP Malpighian tubule level on noninfected locusts when added to saline for 45 min (Fig. 7).

Recovery of the Sensitivity to AVP-like IDH after the Infection

Our infected colony was treated with antibiotics and the infection steadily declined. Microscopic examinations confirmed this decline and as soon as parasites became undetectable in Malpighian tubules a bioassay was carried out. It demonstrated that Malpighian tubules were again responsive to the AVP-like IDH (Fig. 8).

FIG. 1. Trophozoites of *Malameba locustae* in the Malpighian tubules of *Locusta migratoria*. (A) Infected Malpighian tubules with damaged brush border and ruptured wall cell (►). (B) Normal Malpighian tubules from noninfected locust. (C) Rectal pad from infected locust (×125).
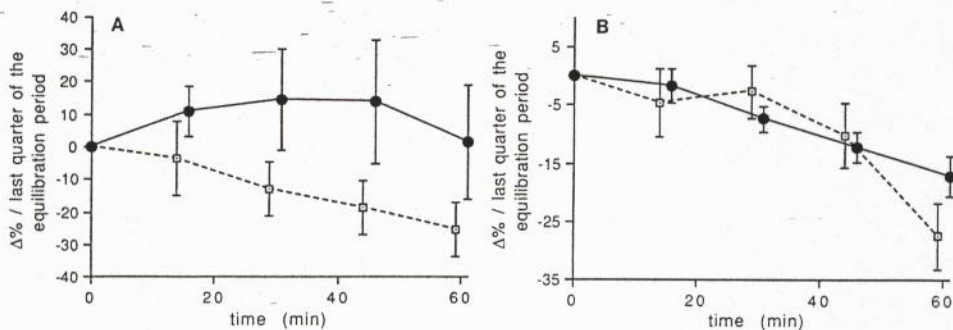
FIG. 2. Excretion of primary urine of infected Malpighian tubules receiving crude (A) or prepurified (B) diuretic extracts of thoracic ganglia. Control (□), stimulated with two-ganglia equivalent (●); mean ± SEM, $n = 6$ in each group.

DISCUSSION

The protozoan infection caused by *Malameba locustae* deeply alters the Malpighian tubules structures. The lumen is totally invaded by spores which cause a distension leading, in some cases, to a tearing of the tubule. In contrast, the rectum is not affected and its structure remains intact.

Infected animals are no longer able to correctly regulate their fluid excretion. Isolated Malpighian tubules do not behave normally. Instead of a quick decline when the hormonal stimulus is lacking (controls) and a prompt increase in diuresis for the hormonal-stimulated locusts (Proux et al., 1988), we observed a slow decrease for controls and little or no increase in

"stimulated" tubules. Only the Malpighian tubules are concerned since (1) the AVP-like IDH content of the ganglia, which is steadily measured in our laboratory, remained stable during the infection (around 3000–4500 pg/mg of protein) and (2) noninfected locusts treated with diuretic extracts prepared from infected locusts are perfectly stimulated and, conversely, infected locusts treated with diuretic extracts prepared from noninfected locust are not stimulated.

It is likely that this lack of stimulation is due to cellular damages affecting the serosal membrane of the tubules and disturbing the hormone–receptor bond. The cytosol remains functional since a cyclic AMP analogue is efficient and the phosphodi-
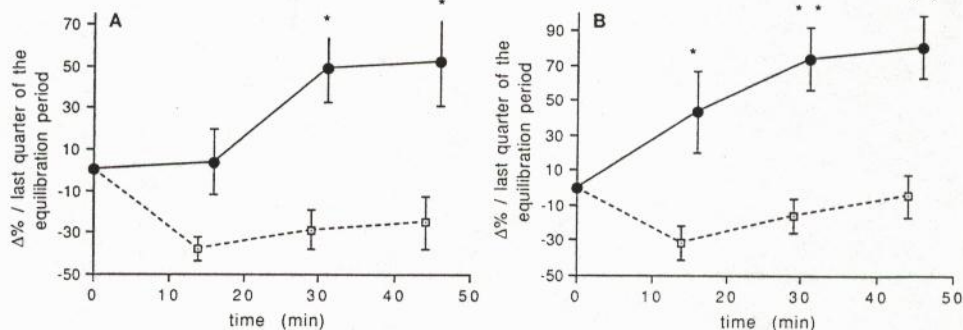

FIG. 3. Excretion of primary urine of infected (□) and noninfected (●) Malpighian tubules receiving crude extracts of thoracic ganglia prepared from infected (A) or noninfected (B) locusts. Mean ± SEM; $n = 5$ or 6 for each group: *,**Significant ($P < 0.05$) and very significant ($P < 0.01$) difference between the groups.

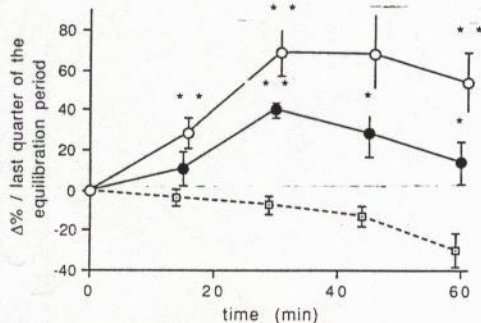FIG. 4. Excretion of primary urine of infected Malpighian tubules receiving saline (□) or 4.10^{-5} M CTP cyclic AMP (●) and 10^{-5} M CTP cyclic AMP (○). Mean ± SEM; $n = 5$ for each group. *·**Significant ($P < 0.05$) and very significant ($P < 0.01$) difference between each stimulated group and the control group.

FIG. 6. Excretion of primary urine of infected Malpighian tubules receiving saline then 10^{-5} M CTP cyclic AMP (□) or 10^{-4} M forskolin + 2.10^{-4} M IBMX then 10^{-5} M CTP cyclic AMP (●). Mean ± SEM; $n = 9$ and 8, respectively. *Significant ($P < 0.05$) difference between the groups.

esterase activity is present. The inner part of the membrane is also functional since cyclic AMP metabolism is still effective as demonstrated by its stimulation when using forskolin. Although functional on the whole, the membrane is affected by the parasite. The mixture forskolin + IBMX is not very efficient at increasing fluid excretion but causes a huge (50-fold) increase in cyclic AMP tubule concentration. The forskolin and IBMX concentrations used here are identical to those used on noninfected insects where they caused some increase in fluid excretion but "only" a 20-fold increase of the cyclic AMP tubule level (Proux and Hérault, 1988). These concentrations could be too high here. Indeed the damaged serosal membrane could trigger an important entry of forskolin and IBMX leading to a "running away" of the cyclic AMP metabolism, the first consequence of which could be a slowing of the tubule stimulation due to a down-regulation of the cyclic AMP or to a negative effect on other possible second messenger(s). The relatively low stimulation observed in some
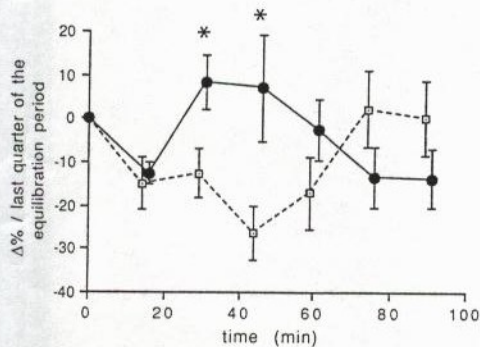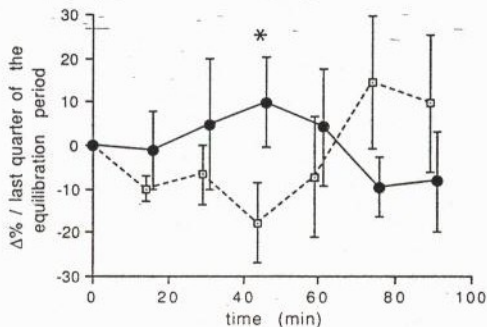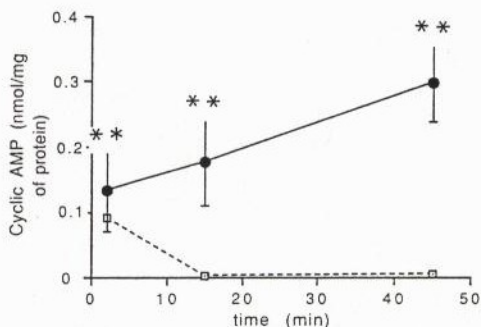

FIG. 5. Excretion of primary urine of infected Malpighian tubules receiving crude extracts of thoracic ganglia (two-ganglia equivalent) then 10^{-5} M CTP cyclic AMP (□) and vice versa (●). Mean ± SEM; $n = 10$ and 12, respectively. *Significant ($P < 0.05$) difference between the groups.

FIG. 7. Effect of saline (□) or 10^{-4} M forskolin + 2.10^{-4} M IBMX (●) in Malpighian tubules endogenous cyclic AMP level as a function of time. Mean ± SEM; $n = 5$ for each group. **Very significant ($P < 0.01$) difference between the groups.
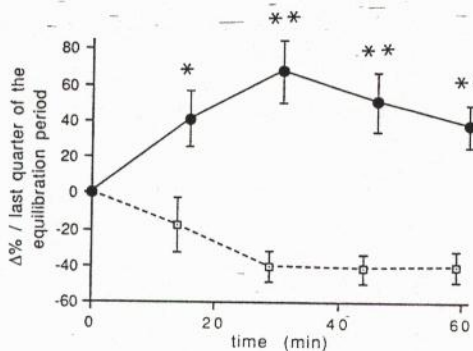
FIG. 8. Excretion of primary urine of noninfected Malpighian tubules from a colony having recovered from an infection and which received crude extracts of thoracic ganglia (two-ganglia equivalent) (●) or saline (□). Mean ± SEM; $n = 5$ for each group. *·**Significant ($P < 0.05$) and very significant ($P < 0.01$) difference between the groups.

cases with the CPT cyclic AMP (particularly with a high concentration) supports this hypothesis. The too important entry of the analogue could cause a partial inhibition of the stimulation. Another hypothesis is the modification of the cyclic AMP metabolism due to the parasite itself.

Although our colony was severely stricken by infection (100% of the locusts were infected, and the mortality was very high), we managed to save it. Having recovered from this infection, the responsiveness to the AVP-like IDH returned to the preinfection levels.

It must be stressed that the rectum, the other organ of diuresis, had never been affected by the parasite during the infection. Its sensitivity to the antidiuretic substance as well as its second messenger metabolism remained unchanged (Fournier, pers. commun.).

This sensitivity of the locust to *Malameba locustae* is of primary importance. A survey of every locust colony is essential and can be easily done either visibly or by microscopic examination of some Malpighian tubules. In addition, a precise study of the way the parasite affects the working of Malpighian tubule could be of interest in

the frame of pest control since (1) only Acrididae seem to harbor *Malameba* (Martoja, 1969) and (2) infections were reported for several locust species collected in the field (Henry, 1968).

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