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Effects of lignoids on a hematophagous bug, *Rhodnius prolixus*: feeding, ecdysis and diuresis

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Abstract

The effects of lignoids on feeding, ecdysis and diuresis in fourth-instar larvae of *Rhodnius prolixus* (Hemiptera) were investigated. Up to 100 μg/ml burchellin, podophyllotoxin, pinoresinol, sesamin, licarin A, or nordihydroguaiaretic acid (NDGA) in the diet did not induce antifeedant effects. Pinoresinol and NDGA significantly inhibited ecdysis. In experiments in vivo, burchellin and podophyllotoxin reduced the production of urine after feeding. 5-Hydroxytryptamine (5-HT), a diuretic hormone, partially counteracted this effect of burchellin. In experiments in vitro, using isolated Malpighian tubules, (i) burchellin reduced diuretic hormone levels in the hemolymph but not the amount of diuretic hormone stored in the thoracic ganglionic masses (including axons), (ii) burchellin decreased the volume of urine secreted by isolated Malpighian tubules, and (iii) 5-HT could not overcome the effect of burchellin upon the Malpighian tubules. We conclude that burchellin interfered with the release, but not with the production of diuretic hormone by the thoracic ganglionic mass or induced an antidiuretic hormone and directly affected the Malpighian tubules.

Keywords: Lignans; Neolignans; Lignoids; *Rhodnius prolixus*; Feeding; Ecdysis; Diuresis

1. Introduction

The co-evolution of plants and insects has undergone a powerful plant defensive strategy based on phytochemicals with hormonal, antihormonal or toxic activities. A variety of compounds found in plants, often called secondary chemicals, interfere with specific physiological functions, including insect development, reproduction, feeding and behavior. Bowers (1976) and Bowers et al. (1976), for instance found in *Ageratum houstonianum* two compounds with anti-juvenile hormonal activities derived from chromene, 7-methoxy-2,2-dimethyl-3-chromene and 6,7-dimethoxy-2,2-dimethyl-3-chromene, designated precocenes 1 and 2, respectively, which induce precocious metamorphosis in immature insects and inhibit reproduction in adults. The neem tree *Azadirachta indica* contains the highly oxygenated triterpene azadirachtin (Butterworth and Morgan, 1968), which is an insect growth inhibitor (Schmutterer and Rembold, 1980).

Following these biorational approaches and after developing suitable bioassays, we tested compounds from plants on the hematophagous insect *Rhodnius prolixus*, a vector of *Trypanosoma cruzi*, the etiological agent of Chagas' disease. Using this insect as an insect model, we demonstrated that feeding of *R. prolixus* larvae is drastically reduced when precocene 2 is added either to blood or an artificial diet. However, precocene 1 and 7-ethoxyprecocene appear to have only a modest anti-feeding effect (Azambuja et al., 1982) although the latter has considerably more potent antihormonal effects than precocene 2 in suppressing juvenile hormone secretion in *Rhodnius*. Treatment of *Rhodnius* larvae with 7-ethoxyprecocene severely retards moulting or initiates a condition of permanent moulting inhibition; these effects are reversed by a subsequent treatment with ecdysone (Garcia et al., 1984a). Azadirachtin A exerts

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only a slightly antifeeding property but it also has a potent antimoulting activity: in low doses, azadirachtin inhibits ecdysis by its ability to reduce the release/production of prothoracicotropic hormone (PTTH), thereby inducing a decrease of ecdysone levels in the hemolymph of *R. prolixus* (Garcia et al., 1984c, 1986, 1990). These results emphasize that the antifeeding and antihormonal activities of precocenes and azadirachtins are distinct, and that the observed antihormonal effects do not result from inadequate nutrition.

One of the plant compounds, azadirachtin, not only affects the triatomines but also the development of *T. cruzi* in these vectors (summarized by Garcia and Azambuja, 1991; Kollien and Schaub, 2000). Even if it could never be used as a direct treatment against *T. cruzi* because it has to be fed in a mixture with blood, the mechanisms should be clarified. If a topical treatment is possible, such compounds offer the greatest promise for the future control of the transmission of Chagas disease.

Both the abundance of the widely distributed natural plant compounds, the lignoids — lignans and neolignans — (Gottlieb and Yoshida, 1989, 1990), and the reports that these substances have some activities on insects as feeding deterrents or larval growth inhibitors (Harmatha and Nawrot, 1984; MacRae and Towers, 1984; Miyazawa et al., 1994) initiated detailed investigations on the effects of lignoids on triatomines and the interaction with *T. cruzi* (Cabral et al., 1995, 1999, 2000a,b,c). We summarize here these experiments on the effects of lignoids on feeding, ecdysis and excretion in *R. prolixus* larvae.

2. Results and discussion

2.1. Effects on feeding

In different insects, e.g. storage pests, treatment with lignans and neolignans reduces the feeding (Harmatha and Nawrot, 1984; MacRae and Towers, 1984). After supplementation of the blood meal of *R. prolixus* with different concentrations of six lignoids, the effective doses (ED_{50}) were higher than 100 μg/ml of blood meal (Cabral et al., 1995, 2000a) (Table 1). Therefore, burchellin, pinoresinol, sesamin, licarin A, nordihydroguaiaretic acid and podophyllotoxin do not inhibit feeding of fourth-instar larvae of *R. prolixus*. Although podophyllotoxin is a lignan with strong feeding deterrent effect and toxicity in other insects (Harmatha and Nawrot, 1984; MacRae and Towers, 1984), an oral administration of 100 μg/ml of this compound did not induce any antifeedant effect, but it did cause high mortality rates in *R. prolixus*, and killed 33% of the bugs at a dose of 10 μg/ml (Cabral et al., 2000a). Pinoresinol presented also only low toxicity when applied at a dose of 500 μg/ml (data not shown).

2.2. Effects on ecdysis

Whereas complete ecdysis occurred in the control group that received solvent only, the most significant ecdysis inhibition occurred in the group treated with podophyllotoxin (Table 1) (ED_{50} of 10 μg/ml). However, a high mortality rate was observed in this group (>80%), indicating a generalized toxicity (Cabral et al., 2000a). Ecdysis stasis with low toxicity (less than 12%) was obtained with pinoresinol (ED_{50} of 60 μg/ml) and NDGA (ED_{50} of 20 μg/ml) (Table 1). Burchellin, sesamin and licarin A applied orally did not inhibit moulting. Topical and continuous contact treatments with lignans and neolignans induced no significant difference in the percentage of ecdysis of *R. prolixus* larvae (Cabral et al., 2000a). However, the intermoulting period was considerable extended at doses of 100 μg/insect or 100 μg/cm^2, respectively, by treatment with sesamin, licarin A, pinoresinol, burchellin and NDGA. The period between ecdysis of some of these larvae was extended to more than 30 days after feeding, whereas control larvae moulted within 16 days (Cabral et al., 2000a).

It is generally accepted that azadirachtin strongly influences insect hormones by interfering with the neuroendocrine system (Rembold, 1987). Garcia et al. (1990) demonstrated that azadirachtin depressed the levels of ecdysteroids in the hemolymph of *R. prolixus*. The analysis of these findings led us to the conclusion that larvae treated with azadirachtin diminished the synthesis and release of PTTH and consequently ecdysone production. These facts imply the impossibility for the insect to moult to the next instar (Garcia et al., 1986, 1987) and demonstrate that ecdysone therapy overcomes the ecdysial stasis and reduces the moulting delay in *R. prolixus* (Garcia and Rembold, 1984). To test whether the mechanism of NDGA affects ecdysis in a similar way to azadirachtin, we treated a group of 4th-instar larvae

Table 1
ED_{50}[a] (μg/ml) of lignans and neolignans for feeding and ecdysis of 4th-instar larvae of *Rhodnius prolixus*[b]

Compounds[c]	ED_{50} feeding	ED_{50} ecdysis
Burchellin	>100	>100
Podophyllotoxin	>150	10
Pinoresinol	>130	60
Sesamin	>100	>100
Licarin A	>150	>150
NDGA[d]	>100	20

[a] Effective doses inhibiting 50% (ED_{50}) of feeding or ecdysis were determined by computing the linear regression, method of least squares, of the volume of the ingested blood or of the percentage of ecdysis against the dosage of lignoids according to Snedecor (1956).

[b] 10–20 larvae were used at each concentration.

[c] The lignoids were dissolved in acetone–saline (1:4) and added to the blood meal at different concentrations (1, 10, 100 μg/ml).

[d] NDGA, nordihydroguaiaretic acid.

with NDGA (100 μg/ml) and simultaneously with ecdysone (1 μg/ml of blood meal): ecdysone counteracted the moulting inhibition and 100% of the insects moulted to 5th-instar larvae (data not shown). These results indicate direct or indirect interference of NDGA in the neuroendocrine system and emphasize that the PTTH/ecdysone pathway is involved in the mechanism of action of this lignoid (Cabral et al., 2000a).

2.3. Effects on excretion

After blood ingestion, the abdomen of *R. prolixus* is strongly distended, but about 6 h later, the excretory system has removed approximately 50% of the imbibed fluid. It is well known that this subsequent excretion is influenced by several factors such as the previous starvation period and the volume of blood ingested (Wigglesworth, 1972). With both parameters controlled, Cabral et al. (2000a) observed that after treatment with some lignoids the abdomen remained swollen even a week after feeding, indicating an interference with the excretion system. Weighing the insects immediately after feeding and 24 h later, the excretion rates varied after treatment with different lignoids, and podophyllotoxin and burchellin significantly reduced the excretion. The other tested lignoids did not cause significant differences in the excretion when compared with controls (Cabral et al., 2000a). How, could we explain the blockade of excretion by podophyllotoxin and burchellin? The compounds could act via the hormonal regulatory level of urine formation or via direct effects on the Malpighian tubules. Both possibilities were investigated in detailed experiments in vivo and in vitro (Cabral et al., 2000c).

In the experiments in vivo, insects treated with burchellin excreted only 8 μl of urine whereas the control group released 45 μl within 6 h after feeding (Table 2). The inhibition was partially counteracted, when the group treated with burchellin was simultaneously fed on blood containing 5-HT. The volume of urine produced in this group increased to 30 μl, but in the control group was 72 μl (Table 2). Within 24 h control insects excreted a total volume of 51 μl, burchellin treated bugs only 9 μl (Cabral et al., 2000c). Therefore, burchellin apparently acts on the Malpighian tubules or on the production/release of diuretic hormone stored in the thoracic ganglionic masses (including axons), or on both.

Table 2
Cumulative excretion[a] of 4th-instar larvae of *Rhodnius prolixus* within 6 h after feeding on blood, blood containing burchellin, and blood containing burchellin and 5-hydroxytryptamine (5-HT)

Experiments	Excreta[b] (μl)
Control	45.0±17.1
Control + 5-HT	72.2±10.4
Burchellin	8.0±14.6
Burchellin + 5-HT	30.0±14.8

[a] Measured as described in Experimental.
[b] Mean ± S.D. of 10 insects.

In the experiments in vitro, we examined the ability of hemolymph from burchellin-treated and control insects to stimulate secretion by isolated Malpighian tubules obtained from starved insects (Cabral et al., 2000c). Within 15 min hemolymph from fed controls induced secretion of a significantly higher volume of urine (7.3 nl/mm tubule) than the hemolymph obtained from burchellin-fed insects (0.2 nl) (Table 3). The addition of 5-HT after that time increased the rate of urine flow in Malpighian tubules from control (13.9 nl) and treated insects (3.0 nl). These findings support the previous results obtained in experiments in vivo, which indicated that 5-HT counteracted the inhibitory effect of burchellin on diuresis production by *R. prolixus* (Table 2), and that the level of diuretic hormone in the hemolymph was reduced in the burchellin-treated insects.

Therefore, we also measured the secretion rates of isolated Malpighian tubules obtained from insects treated with burchellin and incubated for 15 min in Ringer's solution plus 5-HT (Cabral et al., 2000c). These Malpighian tubules from treated insects secreted only 1.1 nl/mm tubule (Table 3). Additionally, secretory activity of these preparations could not be counteracted by the addition of 5-HT for the subsequent 15 min (0.2 nl/mm tubule). It seems, therefore, that burchellin also can affect the Malpighian tubules' secretion directly. This was verified by an addition of burchellin to secreting Malpighian tubules, which then produced in 15 min less urine (11.8±16.9 nl/mm tubule) than in the incubations without burchellin (22.7±17.5 nl/mm tubule) (Cabral et al., 2000c).

Finally, we investigated whether burchellin affects the production of diuretic hormone (Cabral et al., 2000c). The diuretic activity after addition of the homogenates of ganglionic masses and axons obtained from burchellin-treated and control insects indicated that the tissue homogenates from control and treated groups did not differ significantly in their capacities, inducing diuresis rates of 4.3 nl and 3.4 nl/mm tubule in 15 min experiments, respectively (Table 3). Based on these results, we concluded that production/storage of diuretic hormone by the ganglionic masses and axons was unaffected in the group treated with burchellin.

The present results suggest that, in addition to burchellin action on physiological processes regulated by the ecdysone, it can be involved in the disruption of physiological processes dependent on diuresis regulation. The effect of burchellin on production of urine in vivo was confirmed by our studies in vitro with different combinations of 5-HT, ganglion homogenates, hemolymph and Malpighian tubules originating from treated and control insects, which indicated that burchellin may affect the release of diuretic hormone by the thoracic ganglionic masses (including axons). In addition,

Table 3
Effects of burchellin in vitro on diuresis[a] of isolated Malpighian tubules (MT) of 4th-instar larvae of *Rhodnius prolixus* suspended in hemolymph from controls and burchellin-treated insects or on homogenates of thoracic ganglionic masses (including axons)

MT taken from	MT suspended in	Volume of urine (nl/mm tubule)	
		0–15 min	15–30 min[c]
Starved insects	Hemolymph from fed control insects	7.28 ± 5.02[b]	13.85 ± 11.13
Starved insects	Hemolymph from treated insects	0.21 ± 0.34	2.97 ± 1.97
Treated insects	Ringer's solution plus 5-HT	1.10 ± 1.15	0.21 ± 0.36
Starved insects	Tissue homogenate from fed control insects	4.28 ± 3.03	NT[d]
Starved insects	Tissue homogenate from treated insects	3.35 ± 2.22	NT

[a] Measured as described in Experimental.
[b] Mean ± S.D. (n = 2–8 MT).
[c] Addition of 5-HT at the beginning of this period.
[d] NT, not tested.

burchellin may also directly affect the Malpighian tubule secretion. As an alternative to burchellin action on diuretic hormone release and Malpighian tubule secretion, the reduced urine flow caused by burchellin treatment could be via a release of an antidiuretic hormone. Only a few descriptions of the presence of such a hormone exist, and it was believed that the diminishing of urine production in *Rhodnius* resulted from a decline in diuretic hormone and 5-HT (Maddrell, 1964). However, recently, it was suggested that insect cardioacceleratory peptide 2b (CAP2b) and cyclic GMP are part of the novel mechanism of antidiuresis in *R. prolixus* (Quinlan et al., 1997). The possibility of burchellin being involved in the CAP2b and cyclic GMP system will be studied in future investigations.

3. Experimental

3.1. Phytochemicals

Burchellin was extracted from *Aniba burchelli* (Lauraceae from Amazon region) as reported by Araujo Lima et al. (1972) and was provided by Dr. O.R. Gottlieb (Fundação Oswaldo Cruz, Brazil). Sesamin from *Sesamum indicum* (Pedaliaceae) and licarin A from *Licaria aritu* (Lauraceae) were donated by Dr. M.J. Kato (University of São Paulo, Brazil). Podophyllotoxin from *Podophyllum peltatum* (Podophyllaceae) and nordihydroguaiaretic acid (NDGA) from *Larrea divaricata* (Zygophyllaceae) were purchased from Sigma Chemical Co (St. Louis, MO, USA). Pinoresinol from seeds of *Melia azedarach* (Meliaceae) was extracted as described by Cabral et al. (1995).

3.2. Insects

Fourth-instar larvae of *R. prolixus* were obtained from a longstanding colony reared and maintained in our laboratory (see Azambuja and Garcia, 1997).

3.3. Feeding bioasssay and evaluation of ecdysis and excretion

Insects starved for 30 days were allowed to feed for 30 min on citrated human blood through a membrane feeder (Garcia et al., 1984b). The lignoid samples were diluted in acetone–saline solution (1:4) and added to the blood meal at different concentrations ranging from 1 to 100 μg/ml. Controls received blood with solvent only. Insects were weighed before and immediately after feeding (and 24 h later in the experiments on excretion). Only fully engorged insects were used. The insects were maintained at 28°C throughout the experiments. Moults and mortality were recorded daily until 30 days after feeding/treatment.

In some experiments on excretion of 4th-instar larvae of *R. prolixus*, 1 mM 5-hydroxytryptamine (5-HT), which is considered a second diuretic hormone acting synergistically with diuretic hormones (Maddrell et al., 1991), was added to the blood meal with or without burchellin (100 μg/ml). In addition to weighing, diuresis rates of treated or control insects were evaluated at 28°C during 6 h after feeding and treatment. Urine was collected individually in 1.5 ml Eppendorf tubes (Garcia et al., 1989), and the volume was measured using microcapillaries.

3.4. In vitro diuresis determination

Malpighian tubules were dissected out about 2 h after feeding/treatment or from starved insects and transferred into 20 μl of bathing drops consisting of either hemolymph with or without 1 μM 5-HT or Ringer's solution plus 1 μM 5-HT (Maddrell et al., 1991). The upper tubule part was suspended in the bathing medium, and the cut end secured outside the drop, so that secreted fluid formed a droplet in the paraffin oil (Maddrell et al., 1991). Hemolymph was collected from control and treated larvae 2 h after feeding. In one set of

experiments the prothoracic and mesothoracic ganglionic masses (including axons) were used 2 h after feeding/treatment as source of diuretic hormones (Maddrell, 1963). Tissues from three insects were homogenized (Maddrell et al., 1988), and the homogenate used to stimulate the urine production of isolated Malpighian tubules from treated and control insects. Fifteen min after transfer of the upper Malpighian tubule, the secreted droplet was collected and its diameter measured using an ocular micrometer. The volume of the droplet was calculated using the formula relating the volume of a sphere to its diameter (Maddrell et al., 1991). Secretion volume was determined again 15 min after application of 5-HT. After measurements of secretion in vitro, the length of the tubules was measured and included in the calculation of diuresis according to the formula: rate of diuresis = volume of secreted urine (nl)/length of tubule (mm).

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