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Full Length Research Paper

In vitro cultures of pupal integumental explants to bioassay insect growth regulators with ecdysteroid activity for ecdysteroid amounts and cuticle secretion

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The present study evaluated the in vitro bioassay with pupal integumental explants of the mealworm *Tenebrio molitor* to test the activity of two types of insect growth regulators (IGRs) with ecdysteroidal action. We assessed the imidazole derivative KK-42, that is know as ecdysteroid biosynthesis inhibitor, and the dibenzoylhydrazine RH-0345 (halofenozide) that representing a novel group of IGRs with ecdysteroid agonist action. Two biological endpoint were used, namely the production of ecdysteroid hormone and cuticle secretion *in vitro*. Also the test was done with and without renewment of the culture medium to test the persistence of action of each compound. Essentially, RH-0345 provoked higher ecdysteroid amounts, while KK-42 a reduction. Interestingly when KK-42 was followed by RH-0345, the inhibitory effect was compensated. In parallel, the deposition of cuticle *in vitro* was assessed and it was clear that RH-0345 could induce apolysis premature in the integument explants with the formation of a new cuticle. Here the protein and chitin contents of the explants were tested with RH-0345 and three analogous compounds, RH-2485, RH-5992 and RH-5849.

Key words: *In vitro* bioassay, mealworms, integument, ecdysteroids, cuticle, chitin, proteins**.**

INTRODUCTION

Ecdysteroids and juvenile hormones are the principal hormones regulating moulting, reproduction and diapause in insects (Rees, 1995; Gäde et al., 1997). Previous studies in mealworms, *T. molitor* (L.), showed that ovaries as well as the pupal epidermis are alternative sites of ecdy-steroid production (Delbecque et al., 1990; Soltani-Mazou-ni et al., 1999) and that secretion of a new cuticle can be induced by 20-hydroxyecdysone (20-E) (Soltani et al., 1987, 2002).

In the last three decades, insect growth regulators (IGRs) have shown promise in controlling insects of agricultural, medical and veterinary importance, and in this class, a new group, namely the dibenzoylhydrazines or non-steroidal ecdysteroid agonists, have been developed (Wing, 1988; Dhadialla et al., 2005). Such compounds are hormonally active and disrupt development of pest insects primarily by induction of a precocious and incomplete lethal moulting in several insect orders; they exert their toxicity by binding to the ecdysteroid receptor as does the natural insect moulting hormone, 20-E (Retnaka-ran et al., 1995; Smagghe et al., 2000, 2002; Dhadialla et al., 2005).

In this group of IGRs, a new derivative, RH-0345, showing high toxicity against coleopteran species (Dhadialla et al., 2005), could induce a new cuticle secretion that was similar to the effect of 20-E, and enhanced the ecdysteroid amounts (Soltani et al., 2002; Taïbi et al., 2003). It was striking here that RH-0345 was the most potent (Soltani et al., 2002). In addition the imidazole compound KK-42 is considered as an inhibitor of ecdysteroid biosynthesis. As reported by Kuwano et al. (1983, 1992) and Amrani et al. (2004) KK-42 reduced in adult females the ecdysteroid production by ovaries and

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interfered with reproductive processes. In order to extend previous findings, we examined the *in vitro* activity of RH-0345 and KK-42, applied either alone or in combination, on ecdysteroid production and cuticle secretion *in vitro*. Here the action persistence on ecdysteroid release of each compound tested was evaluated in another experiment with or without renewment of culture medium to test the persistence of action of each compound. In addition, we investigated the effects of RH-0345 on chitin and protein contents in the explants. Interestingly, the effects were compared with three analogues of RH-0345, namely RH-5849, RH-5992 and RH-2485.

MATERIALS AND METHODS

Insects

A continuous stock colony of *T. molitor* was maintained on wheat flour at 27±2°C and 80±5% relative humidity in almost continuous darkness (Soltani-Mazouni et al., 1999). Newly ecdysed *T. molitor* pupae (0 – 4 h) were used throughout the experiments.

Chemicals

The dibenzoylhydrazine compounds RH-0345, RH-5849, RH-5992 and RH-2485 were kindly supplied by Rohm and Haas Research Laboratories (Spring House, PA, USA); KK-42 was kindly provided by Dr. Kuwano (Kyushu University, Japan).

Bioassay using primary integument cultures *in vitro*

Four abdominal sternites from newly ecdysed pupae (0 - 4 h) were isolated, cleaned of extraneous tissue, rinsed three times in a saline solution and incubated for 6 days at 26°C in 0.5 ml of slightly modified Landureau's medium (Soltani et al., 2002). A suitable concentration of RH-0345 and KK-42 (5 µl) was prepared in ethanol, and the compounds were added either alone or in combination to the culture medium at a final concentration of 10 µM. In controls, the culture medium contained 5 µl ethanol per culture. In the combined treatments, explants were also incubated for 6 days with exposure during the first two days in medium containing the first compound alone. After 2 days the second compound was added to the medium without renewment of the culture medium containing the first compound. In another experiment, in order to test the persistence of action of each compound used in combined treatments, the culture medium was renewed at day 2 and as so the explants were incubated for the last 4 days in medium containing the second compound only.

Enzyme immunoassay for ecdysteroids in the culture medium

With integument cultures (as abovementioned), aliquots of 50 µl culture medium were sampled after various times of cultivation (2, 4 and 6 days) and subjected to an enzyme immunoassay (EIA) as previously described (Soltani-Mazouni et al., 1999) using peroxidase as enzymatic tracer, tetramethyl benzidine as a colour reagent and a rabbit B polyclonal antibody. Data are expressed as pg ecdysone equivalents/culture.

Measurements of the new cuticle thickness in integument explants *in vitro*

After 6 days of incubation (as abovementioned), the 6 abdominal integument sternites were removed from medium, fixed in paraformaldehyde (2%) and glutaraldehyde (3%) in cacodylate buffer (0.1 M, pH 7.4) for 12 h at 4°C, and then post-fixed with osmium tetroxyde at 4% for 1 h, dehydrated with ethanol and propylene oxide and embedded in an Epon-Araldite mixture as previously described (Soltani et al., 2002). Semi-thin sections were stained with toluidin blue and the thickness of the pharate adult cuticle, secreted *in vitro*, was measured with a photonic microscope.

Analysis for protein and chitin content in the explants in vitro

After 3 days of incubation, chitin and protein contents in integument explants were determined gravimetrically (Soltani et al., 1996). In this test, next to RH-0345 at 10 µM also three analogous compounds were tested. A suitable concentration of RH-0345, RH-2485, RH-5992 and RH-5849 (5 µl) was prepared in ethanol, and the com-pounds were added to the culture medium at a final concentration of 10 µM.

Each individual sample was washed for 24 h in ether-chloroform (1:1, v/v) and dried to constant weight at 60°C. Then the samples were treated with NaOH (2N) at 100°C for 2 h to remove proteins. The residue obtained was considered to be chitin and was rinsed qui-ckly with ethanol and dried to constant weight. The difference bet-ween the dried constant weight and the chitin content was consi-dered as the protein content.

Statistics

Results are presented as the mean±SD. The age and numbers of samples tested per series are given with the results. Data were subjected to ANOVA and the least significant difference was used to separate means. Homogeneity of variances was controlled by the Levene method (Dagnelie, 1998).

RESULTS

In vitro activity of RH-0345 and KK-42 on ecdysteroid production by pupal integument

Pupal sternal explants incubated *in vitro* released significant amounts of ecdysteroids into the culture medium (Table 1). RH-0345 and KK-42, applied either alone or in combination, were assayed on this ecdysteroid release. The ecdysteroid amounts, determined by EIA at various times of incubation, were subjected to ANOVA. Results of statistical analysis revealed a significant difference ($p<0.001$) between treatments for each time of incubation. When explants were incubated with 10 µM RH-0345 alone, EIA measurements indicated a significant increase of 4- to 6-fold of this hormonal release as compared with untreated controls (Table 1). In contrast, the addition of KK-42 alone caused a significant reduction in the amount of ecdysteroids. In combined treatments, results showed that treatments with RH-0345 followed by KK-42 enhanced significantly the hormonal produc-

Table 1. *In vitro* effect of RH-0345 and KK-42 at 10 µM, applied alone or in combination, on the ecdysteroid amounts (pg/culture) released into the culture medium by pupal integument explants of *Tenebrio molitor* at various times of incubation (m ± sd, n = 4). For each time of incubation, means followed by a different letter are significantly different ($p<0.05$). (R= Renewment of culture medium, NR= no renewment of culture medium).

Treatments	Time of incubation (days)		
	2	4	6
Controls	308 ± 16 b	348 ± 39 b	672 ± 69 c
RH-0345	1196 ± 72 c	2076 ± 167 d	2581 ± 583 d
KK-42	89 ± 8 a	174 ± 25 a	250 ± 32 a
RH-0345+KK-42; R	1198 ± 81 c	2109 ± 10 d	2287 ± 79 d
RH-0345+KK-42; NR	1192 ± 74 c	1522 ± 76 c	1941 ± 185 d
KK-42+RH-0345; R	98 ± 9 a	282 ± 34 b	735 ± 65 c
KK-42+RH-0345; NR	90 ± 6 a	111 ± 60 a	465 ± 66 b

Table 2. *In vitro* effect of RH-0345 and KK-42 at 10 µM, applied alone or in combination in an unrenewed or in a renewed medium, on the thickness (µm) of newly deposited cuticle by integument explants of *Tenebrio molitor* measured after 6 days of incubation (m ± sd, n = 4). Mean values of cuticle thickness followed by a different letter are significantly different ($p<0.05$).

Treatments	New cuticle thickness (µm)	
	Unrenewed medium	Renewed medium
Controls	0.00 ± 0.00 a	0.00 ± 0.00 a
RH-0345	4.10 ± 0.07 b	3.44 ± 1.13 b
KK-42	0.00 ± 0.00 a	0.00 ± 0.00 a
RH-0345+KK-42	0.00 ± 0.00 a	3.30 ± 1.27 b
KK-42+RH-0345	0.00 ± 0.00 a	0.00 ± 0.00 a

tion by pupal epidermis as compared to controls. The renewment of culture medium was also evaluated on ecdysteroid release. When RH-0345 was followed by KK-42, renewment of culture medium generated significant differences at day 4 ($p<0.0001$) as compared to no renewment of culture medium. When KK-42 was followed by RH-0345, renewment of culture medium reduced significantly the negative effects induced by KK-42 on hormonal production as compared to no renewment of culture medium. There were significant differences beteen values recorded at days 4 ($p=0.024$) and 6 ($p=0.006$).

In vitro activity of RH-0345 and KK-42 on cuticle secretion

Integuments were cultured in the presence of RH-0345 or KK-42 added alone or in combination. Their effects have been examined on induction of apolysis and new cuticle deposition (Figure 1). In control sternal explants cultured for 6 days, the integument did not enter apolysis and no new cuticle secretion was observed. In contrast, explants cultured with 10 µM RH-0345 alone did undergo apolysis and subsequently secreted a new cuticle that was pharate adult cuticle. Only explants from series treated with RH-0345 either alone or followed by KK-42, with renewment of the medium, showed symptoms of new cuticle deposition. It was of interest that the thickness of these new cuticles was similar in both cases and averaged 3.6 µm (4.10, 3.44 and 3.30 µm) (Table 2).

In vitro effect of four ecdysteroid agonists on protein and chitin contents of cuticle

Previously, we have shown that the apolysis occurred at day 3 under normal conditions (Soltani et al., 1987). The protein and chitin contents in abdominal explants of *T. molitor* were determined gravimetrically after 3 days of incubation in control and treated series with the four dibenzoylhydrazines. Results presented in Table 3 shows that the four non-steroidal agonists added at 10 µM to

Table 3. *In vitro* effect of four dibenzoylhydrazine compounds, RH-0345, RH-2485, RH-5849 and RH-5992 at 10 µM on protein and chitin contents in integument explants of newly ecdysed pupae of *Tenebrio molitor* after 3 days of incubation treatment. Mean values (m ± sd, n = 4) followed by a different letter are significantly different ($p<0.05$).

Treatments	Protein content (mg)	Chitin content (mg)
Controls	5.34 ± 0.47 a	2.25 ± 0.18 a
RH-0345	5.11 ± 0.32 a	2.75 ± 0.18 b
RH-2485	5.04 ± 0.64 a	3.06 ± 0.30 b
RH-5849	5.18 ± 1.19 a	2.25 ± 0.17 a
RH-5992	5.27 ± 0.23 a	2.14 ± 0.18 a

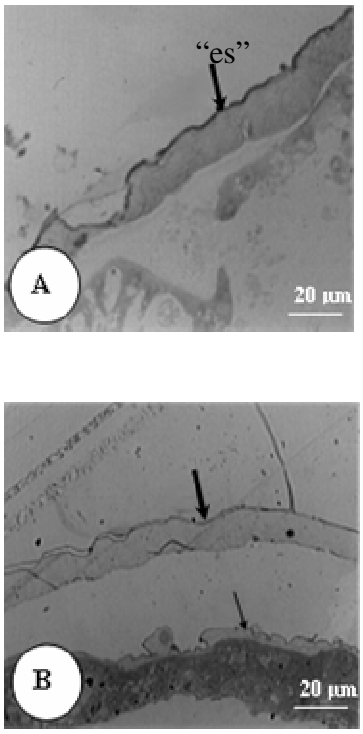

Figure 1. Semi-thin section of the integument of sternite explants originating from 0-day old pupae of *Tenebrio molitor* and incubated for 6 days in untreated medium (**A**) or medium treated with RH-0345 at 10 µM (**B**). In both micrographs, the large bold arrow indicates the presence of the old cuticle. In the explants exposed to RH-0345, the smaller arrow points to the newly deposited cuticle that is present under the large exuvial space (es), confirming the induction of precocious apolysis

the culture medium of integument explants had no significant effect on the cuticular protein content as compared to controls. On the other hand for the chitin content, treatment with RH-0345 and RH-2485 significantly ($p<0.01$) increased the chitin content. The amounts of chitin in the abdominal sternites explants increased from 2.25±0.18 mg in control series to 2.75 ± 0.18 mg in RH-0345- and 3.06 ± 0.30 mg in RH-2485-treated series.

DISCUSSION

IGRs have been reported to possess a specific activity spectrum with a novel insecticidal mechanism not based on a neurotoxic action. They disrupt the physiology and development of target pest insects and show no/low toxicity towards non-target organisms, making them ideal in combination with biological control and also to circumvent insecticide resistance (Wing, 1988; Dhadialla et al., 2005). The ecdysteroid agonist compounds exert their toxicity by binding to the ecdysteroid receptor as does the natural insect moulting hormone 20-E (Dhadialla et al., 2005). Several potential ecdysteroid biosynthesis effectors were tested on the biosynthesis/release of ecdysteroids. Recently, various studies have shown that the ecdysteroid agonist RH-0345 applied alone affects growth and development of oocytes of *T. molitor* (Soltani et al., 1998). Additionally, when applied alone, it enhanced the ecdysteroid release by pupal integument (Soltani et al., 2002) and by ovaries (Taïbi et al., 2003). RH-0345 applied topically on newly ecdysed pupae had not effect on peak position but it caused an increase in hemolymph ecdysteroid titre (Soltani et al., 2002). Also in whole larval bodies of the house mosquito *Culex pipiens*, the ecdysteroid titres increased after topical treatment of larvae *C. pipiens* with RH-0345 (Boudjelida et al., 2005). In adult crickets, *Gryllus bimaculatus,* Lorenz et al. (1995) reported that RH-5992 increased the ecdysteroid synthesis in ovaries. KK-42, an imidazole derivative, was reported to inhibit the moulting hormone synthesis (Kadano-Okuda et al. 1994; Lorenz et al., 1995) and to disturb the growth and the development of oocytes and to reduce the amounts of ecdysteroid released into the culture medium by ovaries (Soltani et al., 1997). In order to extend these previous results, in the current experiments conducted *in vitro* with pupal abdominal sternites of *T. molitor*, RH-0345 was applied alone and in combination with KK-42. Data showed that RH-0345 increased significantly the ecdysteroid release. In contrast, KK-42 alone

caused a significant reduction in the amount of ecdysteroids. RH-0345 appeared to compensate the reduction of ecdysteroid release induced by KK-42, when KK-42 was removed from the culture medium. It was clear that there was no inhibitory action of KK-42 when it was applied after RH-0345, with renewment of the culture medium. The inhibitory action of KK-42 on ecdysteroid production was also observed in prothoracic glands and ovaries under *in vivo* and *in vitro* conditions with *Bombyx mori* (Kadono-Okuda et al., 1987; Shiotsuki et al., 1999), *Locusta migratoria* (Suzuki et al., 1993, Jarvis et al., 1994) and *G. bimaculatus* (Lorenz et al., 1995).

In a second series of experiments the two compounds RH-0345 and KK-42 were evaluated on cuticle secretion. The secretion of a new cuticle in mealworms can be induced by 20-E and also by RH-0345 (Soltani et al., 1987, 2002). Our assays showed that when explants were treated with RH-0345, either alone or followed by KK-42 with renewment of culture medium, a new induced cuticle was observed. The thickness of the secreted cuticle of about 3.6 µm demonstrated new endocuticle formation. The appearance of this newly secreted cuticle *in vitro* and also *in vivo* (Soltani et al., 2002) suggested that the ecdysteroid agonist induced apolysis with the trigger of chitin. Two days of incubation with RH-0345 did not start the onset of apolysis. There seems to be need for a longer incubation to have a successful new cuticle deposition. The amount of ecdysteroids released into the culture medium was not sufficient to induce a new cuticle. Indeed, the value of the ecdysteroid peak detected *in vivo* at the apolysis was 4 µg 20-E equivalents per ml as measured by RIA in hemolymph of mealworm pupae (Soltani et al., 1984). Similarly, RH-0345 applied on newly moulted fourth-instar larvae of *C. pipiens*, increased the thickness of both old and newly cuticles (Boudjelida et al., 2005). Smagghe et al. (2000) have reported that the four dibenzoylhydrazine compounds tested *in vivo* on the last-instars of *Spodoptera littoralis*, caused premature moulting leading to death and that they could initiate and sustain the evagination of isolated wing discs *in vitro*. In the present experiment the abdominal sternites were explanted on the newly ecdysed pupae of *T. molitor* and cultured for 3 days in the culture medium with the four dibenzoylhydrazines. The biochemical composition of the deposited cuticle was determined using a gravimetrical method. Data showed that the chitin content of integumental explants increased significantly only with RH-0345 and RH-2485. Otherwise, there were no significant effects on the cuticular protein content after 3-day of incubation. The same results have been observed when triflumuron (benzoylphenylurea) was injected to newly ecdysed pupae of *T. molitor*, where it reduced the amount of cuticle chitin without any significant effect on protein level (Soltani et al., 1996). Various studies have shown that incubations during 24 h with 20-E enhanced chitin synthesis as measured by incorporation of [^{14}C]GlcNAc in cells of *Plodia interpunctella* and tissues of *Chilo suppressalis*. In a similar manner the ecdysteroid agonist compounds were also able to induce the incorporation of [^{14}C]GlcNAc for a higher chitin deposition (Silhacek et al., 1990; Oikawa et al., 1993)

It has been reported that KK-42 applied alone or with RH-0345 also negatively affected the reproductive events in mealworms. It reduced significantly the oviposition period. Fecundity and egg viability recorded after treatment with RH-0345 alone or in combination with KK-42 were significantly higher than with KK-42 alone (Soltani-Mazouni et al., 2001; Amrani et al., 2004). Topical treatment of *P. interpunctella* with RH-5992 resulted in mortality and the treated females showed small ovaries with fewer eggs (Salem et al., 1997). Smagghe and Degheele (1992, 1994a, b) also reported that RH-5849 and RH-5992 reduced fecundity and/or fertility of several lepidopteran pest species by interfering with ovulation and oviposition. Sun et al. (2003) found that in *Cydia pomonella,* RH-5992 and RH-2485 disturb vitellogenin transport. Farinos et al. (1999) clearly showed the negative effects of RH-0345 on oviposition and egg fertility in two coleopteran species (*Aubeonymus mariaefranciscae* and *Leptinotarsa decemlineata*). Molecular studies revealed that the effects of ecdysteroid agonists on insect molting and reproduction were due to the perturbation of gene expression in the hierarchy cascade of vitellogenesis and/or choriogenesis via the EcR/USP complex (Swevers and Iatrou 1999, 2003; Sun et al., 2003).

In conclusion, the current experiment showed that RH-0345 increased the production of ecdysteroids in mealworm *in vitro* integument cultures. Interestingly, this effect agreed with the induction of new cuticle deposition in the treatments. In combined treatments with KK-42 followed by RH-0345, RH-0345 compensated the reduction of ecdysteroids. This obtained information helps to better understand the effects of dibenzoylhydrazines on growth, development and reproduction of *T. molitor*. This can be of use in the control of coleopteran and other important pest insects because these IGRs exert a new activity and are more ecotoxicological benign compared to classical neurotoxic insecticides. Unfortunately, no exact information is available on the molecular pathways inducing the ecdysteroid producing effects so far. Thus, more research is needed to unravel these new aspects and the mode of action of this new dibenzoylhydrazine derivative.

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