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Receptor affinities and biological responses of nonsteroidal ecdysteroid agonists on the epithelial cell line from *Chironomus tentans* (Diptera: Chironomidae)

Stephanie QUACK, Andreas FRETZ, Margarethe SPINDLER-BARTH and Klaus-Dieter SPINDLER

Lehrstuhl für Hormon- und Entwicklungsphysiologie, Heinrich-Heine-Universität Düsseldorf, Universitätsstrasse 1, D-40225 Düsseldorf, Germany

Phenylhydrazine, RH-compounds, insecticide, *Chironomus tentans*, insect, chitin metabolism, cell differentiation, protein pattern, ecdysteroid, receptor

Abstract. The two nonsteroidal ecdysteroid agonists, RH 5849 and RH 5992 were tested in the epithelial cell line from *Chironomus tentans*. Both compounds induce all effects elicited by 20-OH-ecdysone in this cell line, namely differentiation, arrest of cell growth and regulation of chitin metabolism. Usually, the cell line forms multicellular vesicles consisting of a squamous monolayer. Treatment with ecdysteroids or RH compounds leads to the formation of a stratified columnar epithelium. This differentiation is accompanied by changes in protein pattern. Ecdysteroids and RH compounds also interfere with chitin metabolism. Chitin synthesis is inhibited, whereas synthesis and secretion of chitinolytic enzymes are increased. The efficiency of RH 5849, RH 5992 and 20-OH-ecdysone to evoke the above mentioned responses corresponds with their affinity for the ecdysteroid receptor.

INTRODUCTION

Whereas insecticidal action in animals is influenced by many factors like uptake, availability in the target tissue, metabolic stability etc., which may be the cause of species-specific variations in biological activity, the mode of action in the target cell is the same and can readily be studied in insect cell lines, which are responsive to the effector compound.

The epithelial cell line from *Chironomus tentans* exhibits two characteristic biological responses to ecdysteroids, namely tissue differentiation (Spindler-Barth et al., 1988, 1992, 1994a) and chitin metabolism (Spindler-Barth, 1993; Spindler-Barth et al., 1989, 1994a; Baumeister et al., 1992). These effects can also be induced by the nonsteroidal ecdysteroid agonist RH 5849 ([1,2-dibenzoyl]-1-tert-butyl-hydrazine) in the *Chironomus* cell line (Spindler-Barth et al., 1991). This compound was first described by Wing and coworkers. They demonstrated that RH 5849 could replace 20-OH-ecdysone in *Drosophila* and *Manduca* (Wing, 1988; Wing et al., 1988). Later on both new structurally related compounds and their effects on additional species were investigated and it always turned out that these benzoylhydrazines acted as ecdysteroid agonists (Wing & Aller, 1990; Spindler-Barth, 1992).

We studied the influence of RH-compounds on ecdysteroid-regulated biological responses, because we are interested in cell clones with partial or complete resistance to ecdysteroids and hormone analogues (Spindler-Barth et al., in prep.). In this paper we present some data about chitin degradation. We compare the biological effects of RH 5849, RH 5992 and 20-OH-ecdysone with their affinities to the ecdysteroid receptor in order to establish baseline data on normal responsive cells.

MATERIAL AND METHODS

Cell line

The epithelial cell line, established by Wyss (1982) was kindly provided by Dr M. Lezzi (ETH Zürich) and kept in our laboratory since 1986. Cells were grown at 25°C according to Wyss (1982) and propagated by dissociation of the multicellular vesicles by pipetting and dilution with fresh medium 1 : 10 every 10 to 16 days.

Determination of chitinase activity and protein analysis

Chitinase activity was determined by a microfluorimetric assay essentially as described by Spindler & Spindler-Barth (1994).

Total protein was determined according to Bradford (1976) using bovine serum albumin as standard. Protein patterns were analyzed by onedimensional gel electrophoresis as already described (Fretz et al., 1993). Gels were scanned and digitalized (Sharp JX 325 F6, resolution 600 dpi, greyscale mode), and protein concentrations calculated with an imaging system (Phoretix 1D Analysis, background substraction mode: valley to valley).

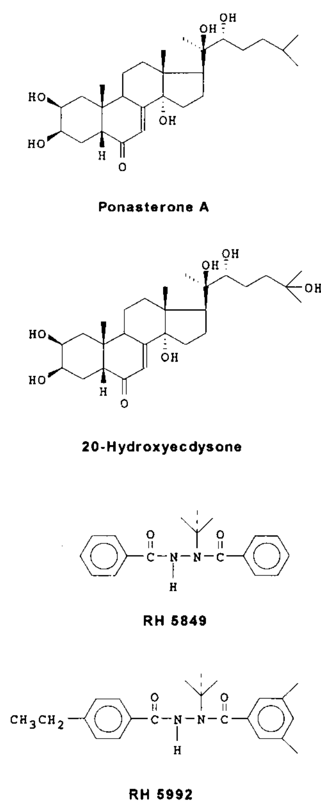

Fig. 1. Structures of ecdysteroids (ponasterone A, 20-hydroxyecdysone) and ecdysteroid-agonists (RH 5849, RH 5992).

Ecdysteroid binding assays

Cells were harvested by centrifugation (13 000 g, 4°C, 20 sec), washed with phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM K_2HPO_4, 1.5 mM KH_2PO_4, pH 6.7) and resuspended in HEPES-buffer (20 mM, 400 mM NaCl, 20% glycerol, 0.1 mM EDTA, 0.5 mM 2-mercapto-ethanol, pH 7.9, freshly supplemented with the following protease inhibitors: 1 µg/ml aprotinin, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin (final concentrations). All operations were carried out on ice except where indicated otherwise. Cells were homogenized with ten strokes in an all glass homogenizer. NaCl was included in the homogenization buffer to extract ecdysteroid receptors from nuclei, since more than 85% of the receptor is already bound to chromatin even in hormonally naive cells (Quack, unpublished observation). The homogenate was centrifuged at 2°C at 100,000 g for 65 min and lipids thoroughly removed. The supernatant containing the cytosolic preparation was immediately desalted with PD-10 columns, (Pharmacia, Freiburg, FRG) since the ecdysteroid binding capacity of the receptor decreases after prolonged incubation under high salt conditions (Turberg et al., 1988).

Ecdysteroid binding was measured by a filter assay using nitrocellulose membranes (Schleicher and Schuell, BA 85) as described earlier (Turberg & Spindler, 1992). Receptor preparations were incubated for 3 hrs at 4°C with 2 nM [^{3}H]-ponasterone A (spec. act. 170 Ci/mmol) without or with 20-OH-ecdysone or the corresponding RH-compounds in various concentrations. All tests were performed in triplicate.

RESULTS AND DISCUSSION

Moulting hormones and ecdysteroid agonists (Fig. 1) induce specific morphogenetic effects in the epithelial cell line from *Chironomus tentans* as already described at the light and electron microscopic level (Spindler-Barth et al., 1992, 1994a). The squamous

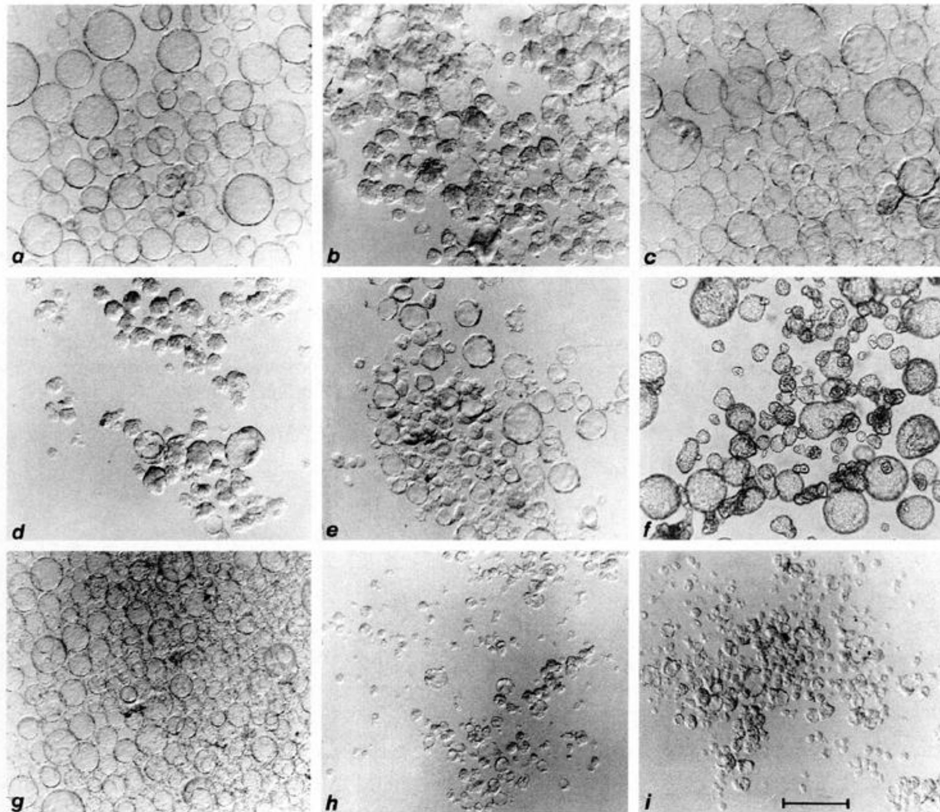

Fig. 2a–f. Influence of 20-OH-ecdysone, RH 5849 and RH 5992 on multicellular vesicles and inhibition of vesicle formation in freshly dissociated cell suspension of the *Chironomus tentans* cell line after incubation with the hormone or agonists for 4 days. Multicellular vesicles were dissociated by pipetting. The bar represents 300 μm. a – multicellular vesicles, control; b – multicellular vesicles, 1 μM 20-OH-ecdysone; c – multicellular vesicles, 50 nM 20-OH-ecdysone; d – multicellular vesicles, 50 nM RH 5992; e – multicellular vesicles, 10 nM RH 5992; f – multicellular vesicles, .1 μM RH 5849; g – dissociated cells, control; h – dissociated cells, 1 μM 20-OH-ecdysone; i – dissociated cells, 100 nM RH 5992.

monolyer of the multicellular vesicles changes under the influence of molting hormones to a stratified columnar epithelium. This morphogenetic effect is ecdysteroid-specific (Spindler-Barth et al., 1994b) and can also be evoked by RH 5849 (Fig. 1) (Spindler-Barth et al., 1991). RH 5992 (Fig. 1) elicits the same effect as ecdysteroids and RH 5849, but at much lower concentrations (Fig. 2). This compound causes an effect already at 10 nM, whereas with 20-OH-ecdysone at least a 10-fold higher concentration is necessary to see first signs of hormonally induced differentiation. Even higher concentrations of RH 5849 are necessary. Maximal response is reached at about 0.1 μM RH 5992 and at 1 μM 20-OH-ecdysone (Fig. 2).

The changes in cell shape and cell arrangement are accompanied by a steep decrease in cell proliferation as measured by thymidine incorporation (Spindler-Barth et al., 1994b).

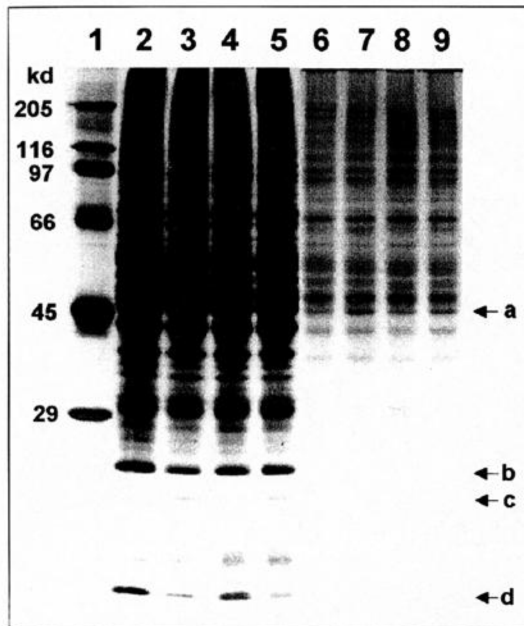

Fig. 3. SDS-PAGE of soluble proteins from the epithelial cell line of *Chironomus tentans* after treatment with 20-OH-ecdysone, RH 5849 or RH 5992 for 4 days. Lane 1 – marker proteins; lanes 2, 6 – control; lanes 3, 7 – 1 µM 20-OH-ecdysone; lanes 4, 8 – 2 µM RH 5849; lanes 5, 9 – 0.4 µM RH 5992. 40 µg protein were applied in lanes 2 to 5, and 10 µg in lanes 6 to 9. a–d: proteins, whose concentrations change after application of the hormone or agonists.

This can be demonstrated most clearly with freshly dissociated cells, in which RH 5992 not only evokes morphological changes in vesicles, but also impairs vesicle formation by inhibiting cell proliferation (Fig. 2). Again, about one order of magnitude higher concentrations are necessary for 20-OH-ecdysone as compared to RH 5992 (Fig. 2). The same parameters, namely induction of specific morphogenetic events and inhibition of cell proliferation have already been used as a criterion for the agonistic effect of RH 5849 on *Drosophila* K_c-cells (Wing 1988).

TABLE 1. Quantitative evaluation of the effects of 20-OH-ecdysone, RH 5849 and RH 5992 on the density of some selected protein bands separated by one-dimensional SDS-PAGE and visualized with silver staining (see Fig. 3).

Band from Fig. 3	Apparent mol. weight Kda	20-OH-ecdysone (1 µM)	RH 5849 (2 µM)	RH 5992 (1.4 µM)
		% of the control		
a	42	222	108	223
b	23	75	87	80
c	22	526	284	415
d	17	30	108	29

The morphological response of *Chironomus* cells is accompanied by an altered expression of proteins after addition of 20-OH-ecdysone (Fretz et al., 1993). This is already visible on one-dimensional PAGE-gels (Fig. 3). Most prominent changes are in protein bands with apparent masses of about 42, 23, 22 and 17 kDa (Fig. 3, Table 1). The concentrations of the 42 and 22 kDa protein bands increase two- and four-to fivefold respectively, whereas the 23 and 17 kDa bands are diminished. Treatment with RH 5992 leads to the same results. They apparently represent a response to receptor saturation, and 20-OH-ecdysone and RH 5992 are equally suitable ligands. If the ecdysteroid receptor is occupied only

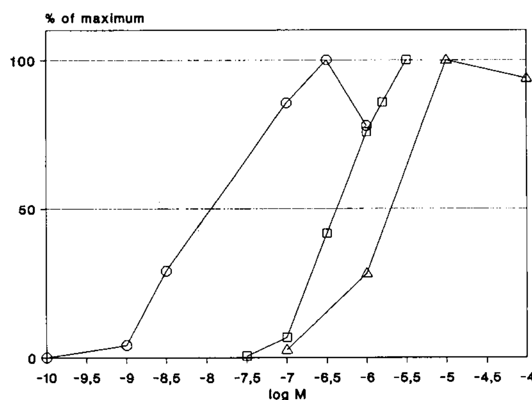

Fig. 4. Effect of RH 5992 (○), 20-OH-ecdysone (□) and RH 5849 (△) on chitinase activity in *Chironomus tentans* cells after incubation for 5 days. (n = 3, S.D. < 10%).

partially (25% saturation with RH 5849 in lanes 4 and 8, Fig. 3) either no or only a weak response is obtained. Also in this case the order of biological activity is RH 5992 >> 20-OH-ecdysone > RH 5849.

RH-compounds also interfere with chitin metabolism. Like ecdysteroids (Baumeister et al., 1992; Spindler-Barth et al., 1989) both substances inhibit chitin synthesis, as already demonstrated for RH 5849 (Spindler-Barth et al., 1991). Again, RH 5992 is superior to RH 5849 (data not shown). The influence of ecdysteroids on chitin synthesis seems to be tissue specific. In body surface epidermis there is a stimulation by molting hormones, whereas in imaginal disc cells chitin synthesis is inhibited in the presence of 20-OH-ecdysone and stimulation occurs only after the hormone level has declined. This type of dual hormonal regulation, typical for imaginal discs (Apple & Fristrom, 1990) could also be shown in *Chironomus* cells for the activity of dopadecarboxylase, another enzyme involved in cuticle formation (Spindler-Barth et al., 1994a).

Table 2. Effects of RH 5849 and RH 5992 and 20-OH-ecdysone on the chitinase activity in relation to their binding to the intracellular ecdysteroid receptor. K_D-values were calculated according to Cheng & Prusoff (1973).

Compound	Ecdysteroid receptor binding		Chitinase activity
	Half-maximal competition* (µM)	K_D-values (µM)	Half-maximal response (µM)
20-OH-ecdysone	1	0.6	0.6
RH 5849	1.8	1.2	2.0
RH 5992	0.02	0.012	0.02

* Binding of [^{3}H]-ponasterone A to the ecdysteroid receptor was competed with nonradioactive RH 5849, RH 5992 or 20-OH-ecdysone.

In contrast to another group of insect growth regulators, the benzoylphenylureas, which also inhibit chitin synthesis but have no effect on chitinolytic enzymes (Londershausen et al., 1989), RH-compounds, like 20-OH-ecdysone (Spindler & Spindler-Barth, in prep.), increase the activity of chitin degrading enzymes in the *Chironomus* cell culture medium. Half-maximal effects are reached at 2 µM RH 5849, 0.02 µM RH 5992, and 0.6 µM 20-OH-ecdysone (Fig. 4, Table 2). This is in accordance with the abilities of these

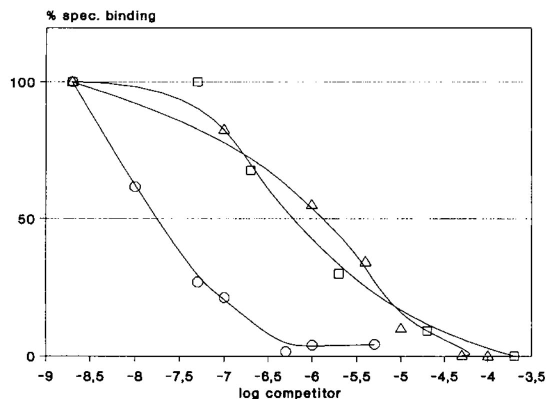

Fig. 5. Competition of ^{3}H-ponasterone A binding to the extracts from *Chironomus tentans* cells with RH 5992 (○), 20-OH-ecdysone (□) and RH 5849 (△). n = 3, S. D. < 10%.

compounds to induce precocious molting in insects and apolysis of spiracular cuticle in isolated abdomens of *Manduca sexta* (Wing et al., 1988; Wing & Aller, 1990).

Both RH compounds compete with [^{3}H]-ponasterone A for binding to the ecdysteroid receptor. Not only the same sequence and dose dependency of efficiency as with all biological responses (Fig. 5, Table 2) including induction of acetylcholinesterase activity in the *Chironomus* cells (Spindler-Barth et al., 1991) are observed, but also the same shape of the curves for [^{3}H]-ponasterone displacement and biological response is obtained (Figs 4, 5). This demonstrates that ecdysteroids not only trigger the biological response but that the receptor occupancy determines the extent of the response. As reported by Terentiou and coworkers (1993) the steepness of the dose-response curve and the ecdysteroid binding curve can vary considerably, despite quite similar K_D- and IC_{50}-values. Wing and coworkers also found a positive relationship between the induction of premature molting in newly ecdysed third instar larvae of *Manduca sexta* and the apparent binding affinity to the ecdysteroid receptor of *Drosophila* K_c-cells for 28 different RH analogs; however, differences in half maximal concentration for receptor binding and biological response were observed (Wing et al., 1988).

The *Chironomus* cell line retains a variety of ecdysteroid regulated responses, and this make it a suitable test system in which both biological response and receptor binding can be measured. Moreover, the characterization of *Chironomus* cell clones resistant to RH-compounds can serve as a model for the development of resistance to hormone mimics (Spindler-Barth et al., in prep.).

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