Action of Brassinosteroids on the Epithelial Cell Line from *Chironomus tentans*

Klaus-Dieter Spindler, Margarethe Spindler-Barth, Andreas Turberg
Institut für Zoologie, Heinrich-Heine-Universität Düsseldorf,
Universitätsstraße 1, D-W-4000 Düsseldorf, Bundesrepublik Deutschland

and

Günter Adam
Institut für Biochemie der Pflanzen, Bereich Naturstoffchemie,
Weinberg 3, D-O-4050 Halle/Saale, Bundesrepublik Deutschland

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The two brassinosteroids, 22*S*,23*S*-homobrassinolide and 22*S*,23*S*-homocastasterone are weak competitors of the binding of [^{3}H]ponasterone A to the intracellular ecdysteroid receptor from the epithelial cell line from *Chironomus tentans*. The relative affinities to the ecdysteroid receptor are 0.001 for both brassinosteroids as compared to 20-OH-ecdysone and 0.1 in comparison to ecdysone. Both substances exert morphological effects similar to those observed with 20-OH-ecdysone. Like moulting hormones both brassinosteroids inhibit chitin synthesis. However, these effects were observed only at rather high concentrations (10^{-5} to 10^{-4} M) which were cytotoxic for 22*S*,23*S*-homobrassinolide.

Introduction

Brassinosteroids are endogenous plant growth regulators with a steroidal structure [1]. The structural similarity of these compounds with ecdysteroids gave rise to several investigations on the biological action of brassinosteroids on insects [2–4] and nematods [5]. In isolated imaginal discs of *Phormia terra-novae* two out of seven tested brassinosteroids had a very weak agonistic effect (only about 15%) and induced evagination at a concentration of 10^{-4} M. All seven compounds, including the two weak agonists, competed with 20-OH-ecdysone in the same assay at concentrations greater than 5×10^{-5} M. It was speculated that both contradictory effects might be due to competition at the ecdysteroid receptor-binding site [2]. Lehmann *et al.* [6] have demonstrated that two brassinosteroids indeed weakly inhibited the binding of [^{3}H]ponasterone A to partially purified ecdysteroid receptor from *Calliphora vicina*. In no instance the biological effect of brassinosteroids and the binding capacity to the ecdysteroid receptor was assayed in the same tissue.

When we examined the influence of the non-steroidal ecdysteroid agonist RH 5849 on the epithelial cell line from *Chironomus tentans* we were able to demonstrate a good quantitative correlation between the ecdysteroid-dependent differentiation and some physiological responses with the ability to bind to the ecdysteroid receptor [7]. We therefore studied the effects of brassinosteroids on the epithelial cell line from *Chironomus tentans,* where both ecdysteroid receptors [7, 8] as well as hormonally regulated processes have been described [7, 9–11]. All these ecdysteroid-dependent effects are regulated in a dose- and time-dependent way.

Reprint requests to Prof. Dr. K.-D. Spindler.

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Materials and Methods

Cells

The cell line which was established by Wyss [12] was kindly provided by Dr. Lezzi (ETH Zürich) and cultured as described earlier [12]. The cells were kept at 25 °C in T-flasks or Erlenmeyer flasks.

Receptor assays

Ecdysteroid receptor assays were performed with cytosolic extracts from *C. tentans* cells as already described [10], with the exception that a different buffer was used for preparation and incubation of cytosolic extracts. Incubation buffer was 20 mM HEPES (pH 7.9), 20 mM NaCl, 20% glycerol, 1 mM EDTA and 0.5 mM 2-mercaptoethanol

freshly supplemented with the following protease inhibitors: 1 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin. As ligand, tritiated ponasterone A (170 Ci/mM) was used.

Acetylcholinesterase

AchE was determined with a microfluorometric assay according to Spindler-Barth *et al.* [9].

Chitin synthesis

Chitin synthesis measured as incorporation of [^{3}H]glucosamine into chitin was studied essentially as described earlier [13, 14].

Protein determinations

Protein determinations were performed according to Bradford [15] using bovine serum albumin as standard. In the case of the AchE assays protein had to be determined according to Lowry *et al.* [16] due to the high detergent content [9].

Results and Discussion

22*S*,23*S*-homobrassinolide and 22*S*,23*S*-homocastasterone (Fig. 1) were used throughout the studies. Both were able to compete for the ponasterone A binding site in *Chironomus tentans* cells, but were much less effective than 20-OH-ecdysone (Fig. 2). In Table I half-maximal competition for the brassinosteroids and two ecdysteroids are given and compared with corresponding values from *Calliphora vicina* [6]. The structures of the two brassinosteroids differ from that of the biologically active ecdysteroid 20-OH-ecdysone: (1) The A/B ring junction is *trans*-fused. (2) The configuration of the vicinal hydroxy groups in the A ring is reversed. (3) Instead of the enone function in ring B both brassinosteroids have a 6-keto and a 7-oxalactone group, respectively. (4) The 14-, 20- and 25-hydroxy groups are missing and an additional hydroxy group is located at position 23. (5) There is an additional ethyl group bound at C24. Ecdysteroids with potent biological activity display a *cis*-fused A/B ring junction and the 6-keto-7-ene group. Positioning of the hydroxyl groups in the side chain is important but changes can be compensated by non-specific interactions [17]. As indicated in the X-ray analysis of 22*S*,23*S*-homobrassinolide [18] the reversed configuration of the 2,3-

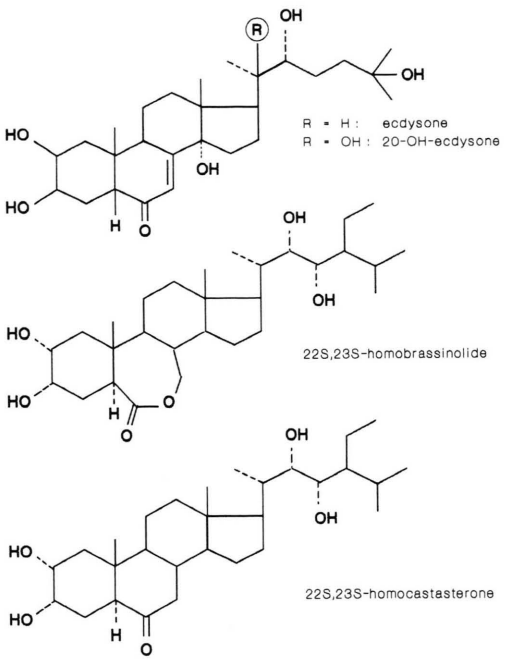

Fig. 1. Structures of 22*S*,23*S*-homobrassinolide, 22*S*,23*S*-homocastasterone, 20-OH-ecdysone and ecdysone.

dihydroxy function of brassinosteroids as compared to ecdysteroids can be compensated to a large extent going from A/B-*trans* to A/B-*cis* fusion. The minor differences in competition of [^{3}H]ponasterone A binding of the two brassinosteroids should be due to the lactone ring structure in

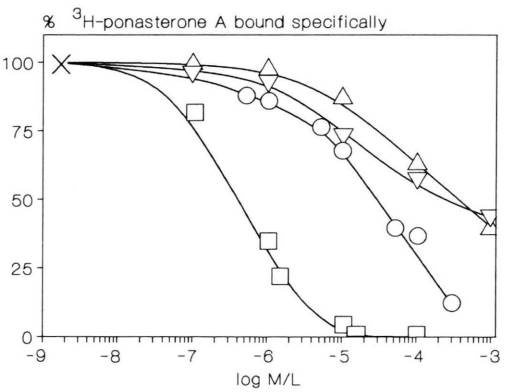

Fig. 2. Competition of [^{3}H]ponasterone A (×) (final concentration 1.7 nM; specific activity 170 Ci/mmol) binding to cytosol from *Chironomus tentans* cells with 22*S*,23*S*-homobrassinolide (△), 22*S*,23*S*-homocastasterone (▽), 20-OH-ecdysone (□) and ecdysone (○). n = 3, S.D. are less than 10%.

Table I. Inhibition of [^{3}H]ponasterone A binding to the ecdysteroid receptor (IC_{50} values). The values for *Calliphora* are calculated from [6].

Steroid	*Chironomus* cells [M]	*Calliphora* [M]
20-OH-Ecdysone	4.0×10^{-7}	2.2×10^{-7}
Ecdysone	3.0×10^{-5}	2.0×10^{-5}
22*S*,23*S*-Homobrassinolide	3.8×10^{-4}	4×10^{-4}
22*S*,23*S*-Homocastasterone	2.4×10^{-4}	4.6×10^{-5}

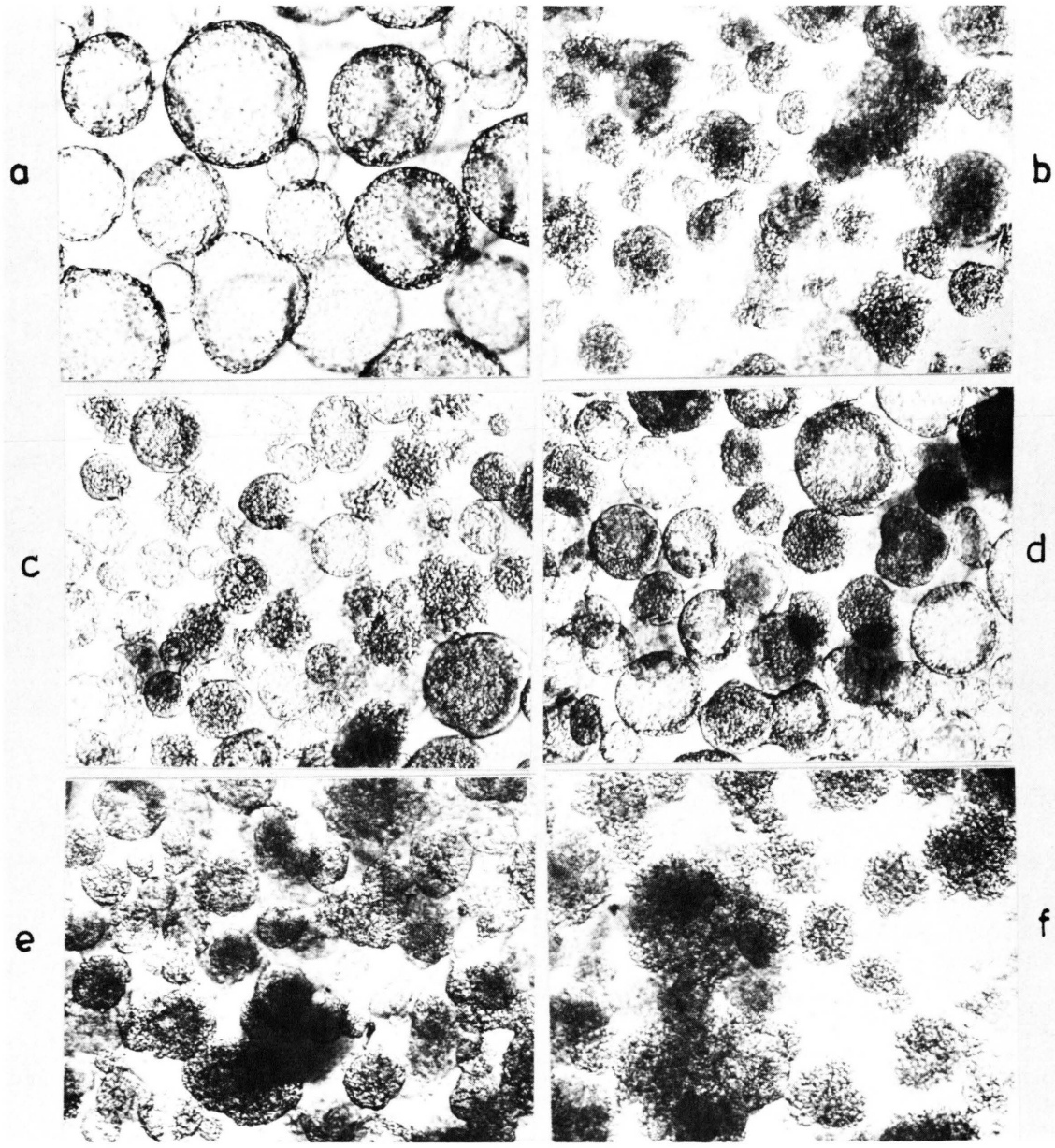

Fig. 3. Influence of 22*S*,23*S*-homobrassinolide (c), 22*S*,23*S*-homocastasterone (d) and 20-OH-ecdysone (b) on the morphology of *Chironomus tentans* cells. a = control. In (e) and (f) homobrassinolide, respectively homocastasterone were applied simultaneously with 20-OH-ecdysone. The two brassinosteroids were used at a concentration of 10^{-5} M, 20-OH-ecdysone at 10^{-6} M, and analyzed after 4 days of incubation. Final magnification: 80 ×.

the 22*S*,23*S*-homobrassinolide. Because of the structural differences outlined above, biological activity of these brassinosteroids should be rather low.

The two brassinosteroids exert a similar morphogenetic effect as 20-OH-ecdysone, but only at rather high concentrations (Fig. 3). In no instance the two brassinosteroids inhibit the action of 20-OH-ecdysone. If given simultaneously with moulting hormone they instead exert a weak additive effect.

Since chitin synthesis, an important process regulated by moulting hormones, is inhibited in the *Chironomus* cells by 20-OH-ecdysone [10], the influence of brassinosteroids on chitin synthesis was studied. Both substances inhibit chitin synthesis as does 20-OH-ecdysone, in a dose-dependent manner. However, about 100-fold higher concentrations of both brassinosteroids are necessary to exert the same effect as 20-OH-ecdysone (Fig. 4). This "agonistic" effect on chitin synthesis confirms the morphological observations (Fig. 3).

The induction of AchE by ecdysteroids was used successfully as sensitive and simple test system for the detection of moulting hormone agonists [19]. This assay is especially suited for the detection of weak agonists or antagonists since there is a considerable increase in enzymatic activity caused by 20-OH-ecdysone, *e.g.* in *Chironomus* cells AchE rises up to 30-fold [9]. However, the influence of both brassinosteroids was erratic although the reproducibility of the enzymatic assay is quite good (<5%). Nevertheless no indication for an antagonistic hormone effect was observed.

The "induction" of AchE by 20-OH-ecdysone is a late event, beginning after 4 days of incubation and reaching a maximum after 7 days. This means that even small toxic effects of the two brassinosteroids may disturb the hormonal response. The determination of the cell density is difficult since the cells grow as multicellular vesicles (Fig. 3). Therefore protein concentration of the cell cultures was determined. The effect of 20-OH-ecdysone and brassinosteroids is most pronounced in cell cultures during the logarithmic phase of cell growth. Under these conditions, 20-OH-ecdysone has only a weak effect on cell growth. The same is also true for 22*S*,23*S*-homocastasterone even at higher concentrations. With 22*S*,23*S*-homobrassinolide there is a dose-dependent effect on protein content. With concentrations even lower than those necessary for half-maximal competition of ponasterone A binding to the ecdysteroid receptor growth of the cells is completely stopped and there is even a loss of protein as compared to the value at the beginning of the treatment; this means that 22*S*,23*S*-homobrassinolide exerts cytotoxic effects (Fig. 5). The main conclusion from our experiments is that in no instance these two steroids are able to prevent or weaken the action of 20-OH-

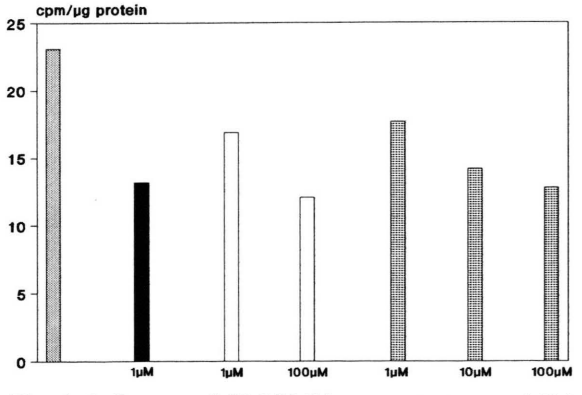

Fig. 4. Influence of 22*S*,23*S*-homocastasterone (▦), 22*S*,23*S*-homobrassinolide (⊓) and 20-OH-ecdysone (■) on chitin synthesis in the *Chironomus tentans* cell line. (▨) = control. (n = 4, S.D. < 12%). The values are given as incorporation of [^{3}H]glucosamine into chitin.

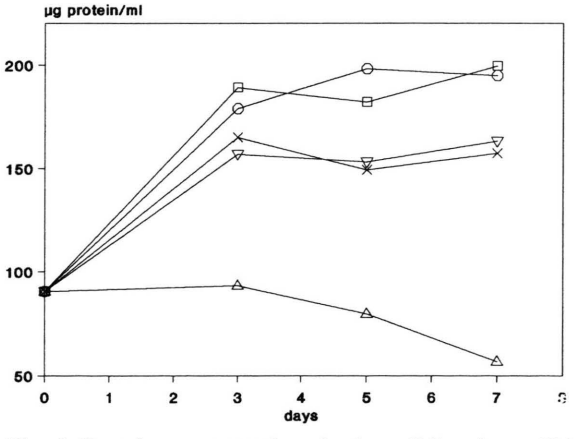

Fig. 5. Protein concentrations in the cell line from *Chironomus tentans*. □ = control, ○ = 20-OH-ecdysone (10^{-6} M), × = 22*S*,23*S*-homocastasterone (10^{-4} M), ▽, △ = 22*S*,23*S*-homobrassinolide (10^{-5} M, respectively 10^{-4} M). The values are means of 3 independent experiments, S.D. < 10%.

ecdysone in this system even in concentrations 100-fold higher than 20-OH-ecdysone. The two brassinosteroids do not exert antagonistic effects in the *Chironomus* cells. Cytotoxic effects of these compounds can lead to misinterpretations as antagonistic or, if moulting hormones inhibit a process like chitin synthesis, as agonistic hormone effects. This might also be the case for the reported antagonistic effect, namely the inhibition of ecdysteroid induced evagination of imaginal discs [4].

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