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ECDYSTEROID RESISTANT SUBCLONES OF THE EPITHELIAL CELL LINE FROM *CHIRONOMUS TENTANS* (INSECTA, DIPTERA). I. SELECTION AND CHARACTERIZATION OF RESISTANT CLONES

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SUMMARY

Chironomus tentans cells were cultured in the presence of gradually increasing concentrations of 20-OH-ecdysone or a nonsteroidal molting hormone agonist, the benzoylhydrazine RH 5992, for a period of about 2 yr. From these cultures, subclones were selected, which are resistant to up to 25 μ*M* 20-OH-ecdysone according to morphological (changes in cell shape and cell arrangement) and physiological criteria (acetylcholinesterase induction, secretion of chitinolytic enzymes, thymidine incorporation). Some subclones, selected in the presence of 20-OH-ecdysone, are resistant only to molting hormone, but still respond to RH 5992 morphologically and biochemically, whereas subclones selected in the presence of the benzoylhydrazine showed no reaction neither to 20-OH-ecdysone nor to the hormone agonist. Hormone resistance is stable; 3 mo. after hormone withdrawal, resistant clones still do not respond to renewed exposure to 20-OH-ecdysone or RH 5992, respectively. Because in all resistant subclones tested so far all hormonally regulated responses known from sensitive cells were no longer detectable, it is assumed that the hormone signaling pathway itself is interrupted. Possible mechanisms of hormone resistance were discussed.

Key words: hormone; resistance; insecticide; benzoylhydrazine; arthropod.

INTRODUCTION

The epithelial cell line from *Chironomus tentans* established by Wyss (1982) has been characterized previously and its response to molting hormones is already extensively described. This cell line is considered as a simplified model to study early events in ecdysteroid-induced tissue differentiation (Spindler-Barth et al., 1989, 1995; Spindler-Barth, 1991, 1993; Lammerding-Köppel et al., 1994) and cuticle formation. The cells are able to produce and degrade chitin. Cell differentiation and chitin metabolism are regulated by ecdysteroids (Spindler-Barth et al., 1989; Spindler and Spindler-Barth, 1994; Quack et al., 1995). Dopadecarboxylase, a key enzyme for sclerotization and tanning of insect cuticle, is also present and modulated by molting hormone in a complex way (Baumeister et al., 1992; Spindler-Barth et al., 1995). The mode of hormonal regulation and the type of morphological and physiological responses of the *Chironomus* cell line resembles the conditions described after hormonal stimulation of imaginal discs from *Drosophila* (Apple and Fristrom, 1990).

The intracellular ecdysteroid receptor present in the epithelial cell line from *Chironomus tentans* is characterized (Turberg et al., 1988) and the gene cloned recently (Imhoff et al., 1993). Generally, there is a good correlation between ecdysteroid receptor affinity of various ecdysteroids, as well as the benzoylhydrazines RH 5849 and RH 5992 and the hormonally induced responses (Spindler-Barth et al., 1991; Quack et al., 1995). These nonsteroidal molting hormone agonists, which are used as insecticides now, elicit all effects induced by molting hormone in this cell line (Spindler-Barth, 1992; Quack et al., 1995).

Stable ecdysteroid-resistant clones are considered as suited negative controls for studies on the molecular mechanism of hormonal regulation on the cellular level. Resistant clones with defects or considerably reduced expression of the ecdysteroid receptor can also be used as homologous expression system for the ecdysteroid receptor. This may be important, since the hormonally induced responses depend on the cell type used for ecdysteroid receptor expression. Resistance to hormone mimics is also of interest, because these nonsteroidal molting hormone agonists of the benzoylhydrazine type (Wing, 1988; Wing et al., 1988) are in use as insecticide (Wing and Aller, 1990). Because resistance against juvenile hormone agonists has already emerged (Shemshedini and Wilson, 1990), it seems reasonable to study the mechanism of resistance to ecdysteroid mimics under laboratory conditions, which may help to develop strategies to delay or circumvent these problems under field conditions.

In this report, we describe the selection of stable subclones of the epithelial cell line from *Chironomus tentans* resistant either to 20-OH-ecdysone alone or to both molting hormone and the nonsteroidal hormone agonist RH 5992.

MATERIALS 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 grow exclusively as multicellular spheroids. Cells were cultured in medium according to Wyss (1982) at 25° C and propagated by dissociation of

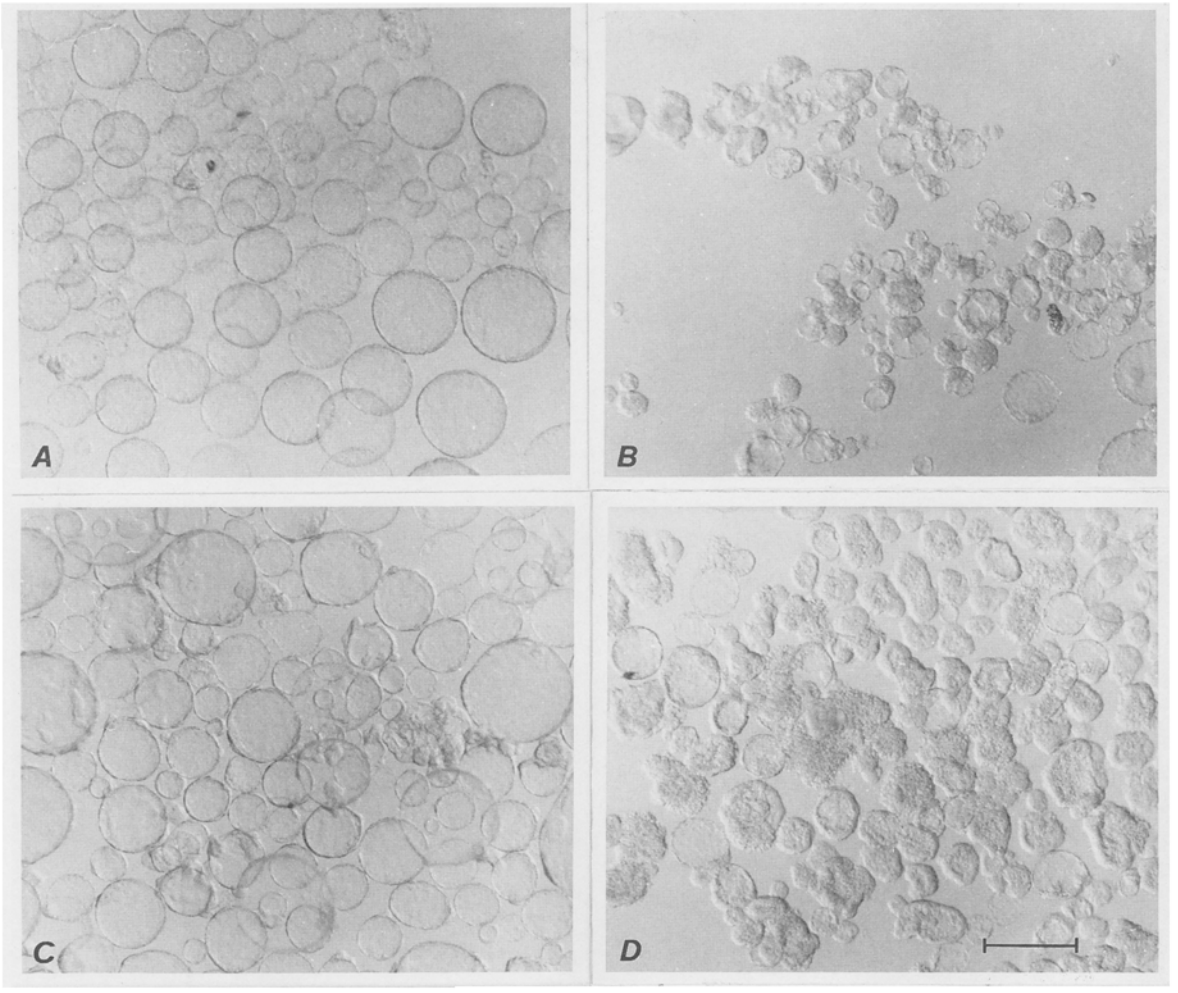

FIG. 1. Influence of 20-OH-ecdysone and RH 5992 on multicellular vesicles of the *Chironomus tentans* cell line after incubation with hormone or agonist for 4 d. The *bar* represents 300 μm. *A* = wild type, control; *B* = wild type, incubated with 1 μ*M* 20-OH-ecdysone; *C* = resistant line ec-r.0; incubated with 1 μ*M* 20-OH-ecdysone; and *D* = resistant line ec-r.0; incubated with 0.1 μ*M* RH 5992.

the multicellular vesicles by pipetting and dilution with fresh medium 1:10 every 10 to 16 d.

Selection of ecdysteroid-resistant cells. According to the method described for *Drosophila* Kc cells (Stevens and O'Connor, 1982), growth inhibition by 20-OH-ecdysone was used to select resistant subclones of the *Chironomus* cell line. Starting from 1 n*M* 20-OH-ecdysone or 0.1 n*M* RH 5992, respectively, gradually increasing amounts of hormone or analogue were added to the culture medium over a period of 2 yr. After this period, individual multicellular vesicles, which originate from single cells by cell division (Spindler-Barth et al., 1992) were picked under microscopical control. The vesicles were then dissociated by pipetting, cells propagated in microtiter plates (24 well, Nunc, Inc.) and recloned. Subclones (n = 45) were considered to be resistant, if a morphological response to ecdysteroids is no longer detectable at a concentration of 5 μ*M* 20-OH-ecdysone or 0.1 μ*M* RH 5992, respectively.

Thymidine incorporation. 7–10 d old cell cultures were dissociated as described above, diluted 1:10 with fresh culture medium, and aliquots of 1 ml transferred to 2.2 ml Eppendorf-cups. To avoid lag phase caused by dissociation of the multicellular vesicles, cultures were incubated for 24 h at 25° C, treated with or without hormone for another 24-h period, and then incubated with ^{3}H-thymidine (Amersham, Braunschweig, Germany, spec. act.: 3,03 TBq/m*M*) for 6 h. Cells were harvested by centrifugation at 10 000 g and washed once with phosphate-buffered saline (PBS) (137 m*M* NaCl, 2.7 m*M* KCl, 8 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, pH = 6.7). The pellet was resuspended in 100 μl PBS and dissolved by addition of 100 μl 0.2 *M* NaOH. An aliquot was taken for protein determination. After precipitation with 5% trichloroacetic acid (final concentration) the pellet was collected on glass-fiber filters (GF/F, Whatman, Maidstone, England) with a vacuum apparatus. Filters were washed three times with 5 ml 5% TCA and three times with 95% ethanol, dried at 40° C for 30 min, and radioactivity determined using a Packard liquid scintillation counter (Tricarb 460C, efficiency 45%).

Determination of enzyme activities. Acetylcholinesterase and chitinase activity were determined by microfluorometric assays essentially as described (Spindler-Barth et al., 1988; Spindler and Spindler-Barth, 1994). Chitin synthesis was measured by incorporation of ^{3}H-glucosamine (Amersham, spec. act.: 1.22 TBq/mmol) into chitin according to Spindler-Barth et al. (1989).

Protein determination. Total protein was determined according to Bradford (1976) using bovine serum albumin (BSA) as standard. In the case of acetylcholinesterase, total protein was determined using the BCA-Protein assay according to the manufacturer (Pierce Chemical, Rockford, IL).

RESULTS

Morphological response. As described previously in detail at the fine structural level (Spindler-Barth et al., 1992), 20-OH-ecdysone induces tissue differentiation, which leads to changes in cell shape and cell arrangement in multicellular vesicles of the hormone-sensitive cell line from *Chironomus tentans* (Fig. 1 *A* and *B*). Resistant

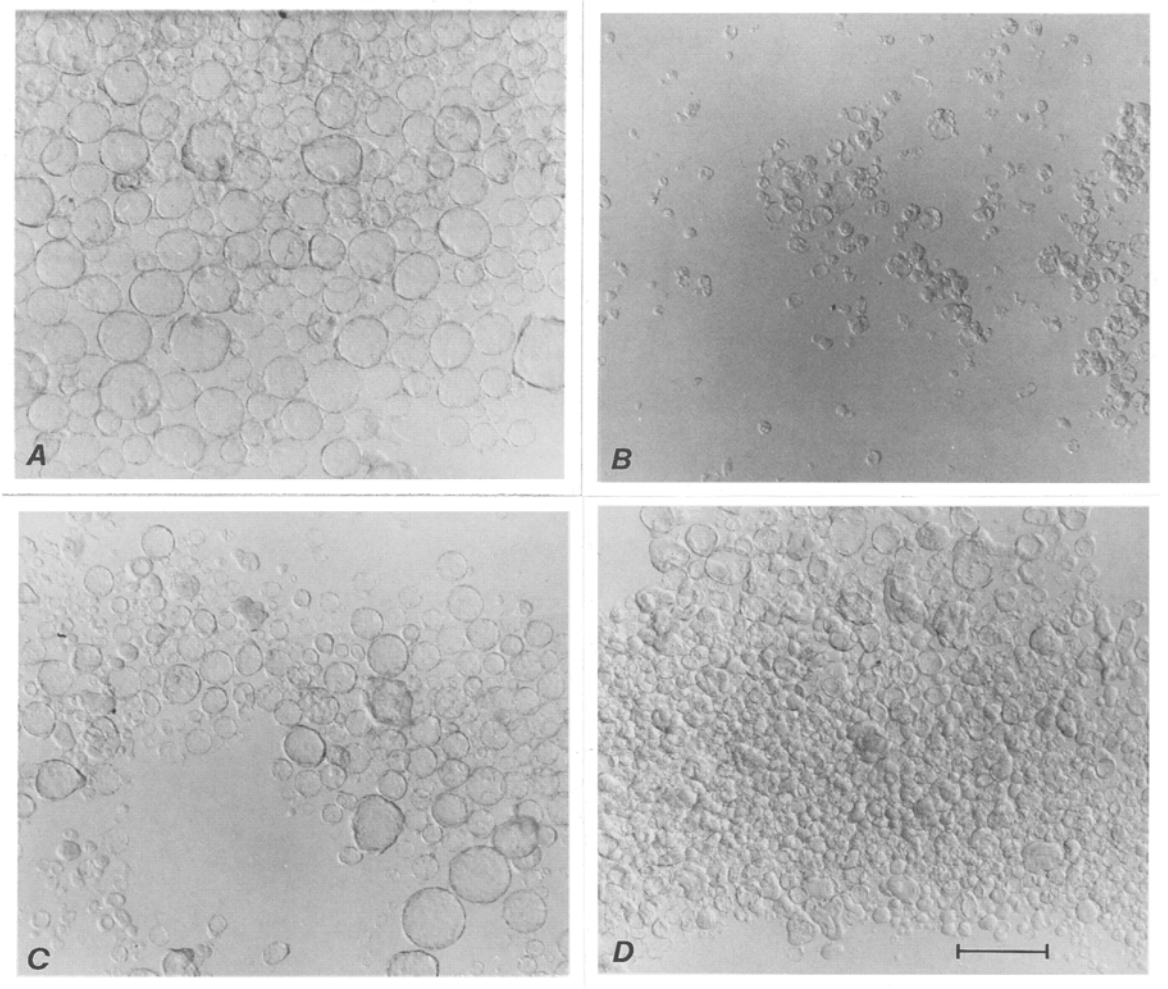

FIG. 2. Inhibition of vesicle formation. Vesicles were dissociated by pipetting. The cells were incubated with hormone or RH 5992 for 4 d. The *bar* represents 300 μm. *A* = wild type, control; *B* = wild type, incubated with 1 μ*M* 20-OH-ecdysone; *C* = resistant subclone ec-r.0, 1 μ*M* 20-OH-ecdysone; and *D* = resistant ec-r.0, incubated with 0.1 μ*M* RH 5992.

subclones grown in the permanent presence of 5 μ*M* 20-OH-ecdysone since 15 mo. showed no morphological response (Fig. 1 *C*), whereas the nonsteroidal molting hormone agonist elicits the usual hormonal-induced reactions (Fig. 1 *D*).

In wild type cells, vesicle formation is also inhibited in the presence of 1 μ*M* 20-OH-ecdysone (Fig. 2 *A* and *B*). Because vesicles arise from single cells by cell division, this indicates that cell proliferation is considerably reduced. As expected, in resistant clones vesicle formation is not impaired by 20-OH-ecdysone (Fig. 2 *C*), but by RH 5992 (Fig. 2 *D*). These results correspond to the data obtained with thymidine incorporation into DNA (Fig. 3). In the sensitive wild type *Chironomus* cell line and in sensitive subclones, DNA-synthesis is depressed by 20-OH-ecdysone in a dose dependent manner, whereas in resistant clones no hormone effect is detectable (Fig. 3).

Effects of 20-OH-ecdysone on enzymatic activities. Because acetylcholinesterase activity is enhanced in the sensitive wild type cell line about 20–30-fold, this enzyme is suited to detect even small residual hormone effects in resistant clones. Whereas in wild type cells a highly significant increase in acetylcholinesterase activity is observed after addition of 1 μ*M* 20-OH-ecdysone, no influence on enzymatic activity is obtained in resistant clones (Fig. 4), even at a concentration of 25 μ*M* 20-OH-ecdysone (data not shown).

Hormone-sensitive subclones, which were cloned by isolation of multicellular vesicles from the wild type cell line, also exhibit differences in hormone sensitivity as shown in Fig. 4 for clone s.0 and s.9. In the most sensitive clone, s.9 acetylcholinesterase activity is stimulated about 40-fold after treatment with 1 μ*M* 20-OH-ecdysone, which is the most effective hormone concentration.

RH 5992 not only elicits the typical morphological responses, induced by ecdysteroids, in the *Chironomus* cell line, but leads also to an increase in acetylcholinesterase activity in the normal range even in those subclones f. e. ec-r.0 and ec-r.2, which were selected for resistance to 20-OH-ecdysone (Fig. 5). As shown previously, RH 5992 is at least 10 times more potent than 20-OH-ecdysone, which corresponds to the higher affinity of the hormone mimic to the ecdysteroid receptor in this cell line (Quack et al., 1995).

The *Chironomus tentans* cell line produces chitinase, which is predominantly secreted into the culture medium (Spindler-Barth,

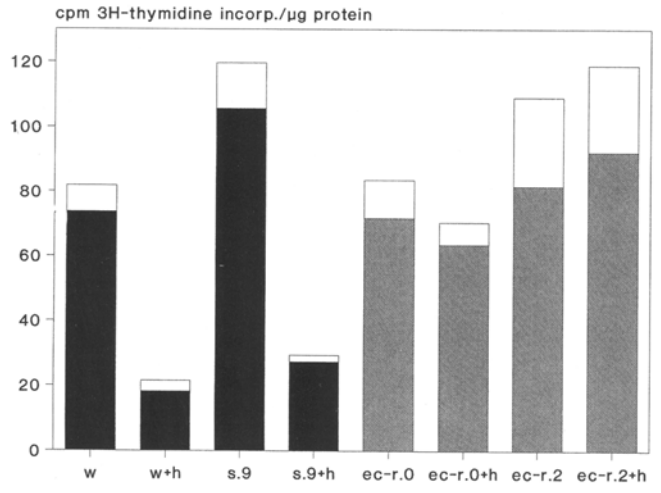

FIG. 3. ^{3}RH-thymidine incorporation into DNA in sensitive cells (■ = wild type, s.9) and resistant subclones (▨; ec-r.0; ec-r.2) of the *Chironomus tentans* cell line in the absence or presence (+h) of 1 µ*M* 20-OH-ecdysone (20E). Cells were incubated for 4 d. Means ± standard deviations (□), n = 3.

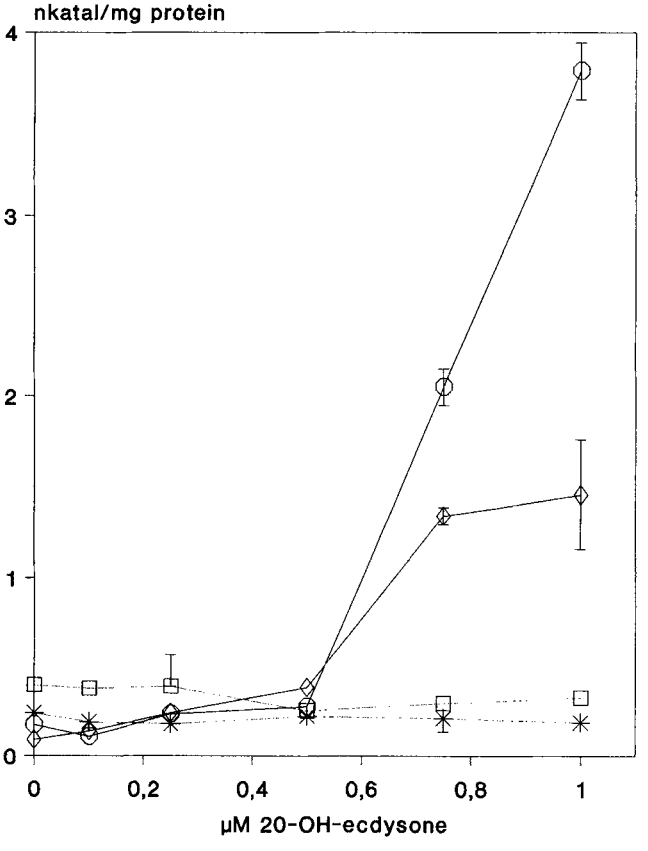

FIG. 4. Dose-response curves showing the influence of 20-OH-ecdysone on acetylcholinesterase activity in wild type and resistant subclones of epithelial *Chironomus tentans* cells. Cells were incubated with hormone for 7 d. ◇ = s.0, ○ = s.9 sensitive clones), □ = ec-r.0, ✳ = ec-r.2 (ecdysteroid-resistant subclones). Means ± standard deviations, n = 6. If no standard deviations are given S. D.s were smaller than the symbols and omitted for clarity.

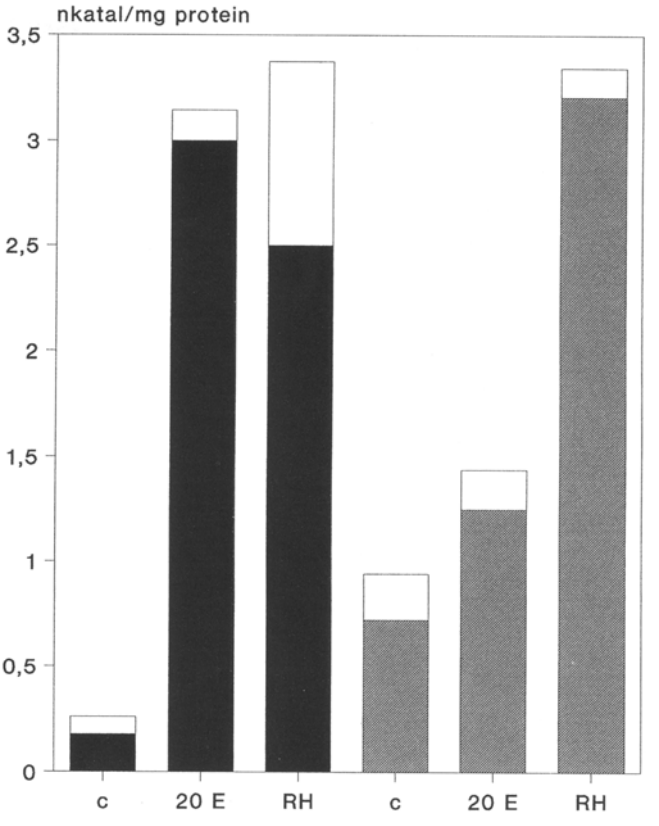

FIG. 5. Influence of 1 µ*M* 20-OH-ecdysone (20E) or 0.1 µ*M* RH 5992 (RH) on acetylcholinesterase activity in sensitive cells (■; wild type) and a resistant (▨;ec-r.0) subclone of the epithelial cell line from *Chironomus tentans*. Cells were incubated with hormone or agonist for 7 d. Means ± standard deviations (□), n = 6.

1993; Spindler and Spindler-Barth, 1994; Quack et al., 1995). After addition of 20-OH-ecdysone, chitinase activity increases in sensitive lines about two- to fourfold. In the ecdysteroid-resistant clones ec-r.0 and ec-r.2, no significant increase is detectable (Fig. 6) even at a concentration of 25 µ*M* 20-OH-ecdysone (data not shown), but chitinase activity is induced after application of RH 5992 (Fig. 7). Due to the weaker response of chitinases to molting hormone, small differences in sensitivity in various sensitive subclones s.0 and s.9 cannot be demonstrated.

Chitin synthesis is inhibited in wild type cells by 20-OH-ecdysone in a dose dependent way (Spindler-Barth et al., 1989). Maximal inhibition is about 50%, which is obtained with 0.1–1 µ*M* 20-OH-ecdysone. Again, chitin synthesis in resistant subclones ec-r.0 and ec-r.2 is not affected by 20-OH-ecdysone (Fig. 8), but inhibition is observed with RH 5992 (data not shown). As is shown in Figs. 1 and 2 and Table 2 the hormonal response to RH 5992 is only partly restored in 20-OH-ecdysone resistant clones.

Resistance towards RH 5992. Using the same procedure as with 20-OH-ecdysone, wild type cells and subclones, which were already resistant to 20-OH-ecdysone (ec-r.), were treated with gradually increasing concentrations of RH 5992. Independent, whether we started with the wild type (rh-r.) or with ecdysteroid-resistant subclones (ec-rh.), we obtained subclones that were resistant against

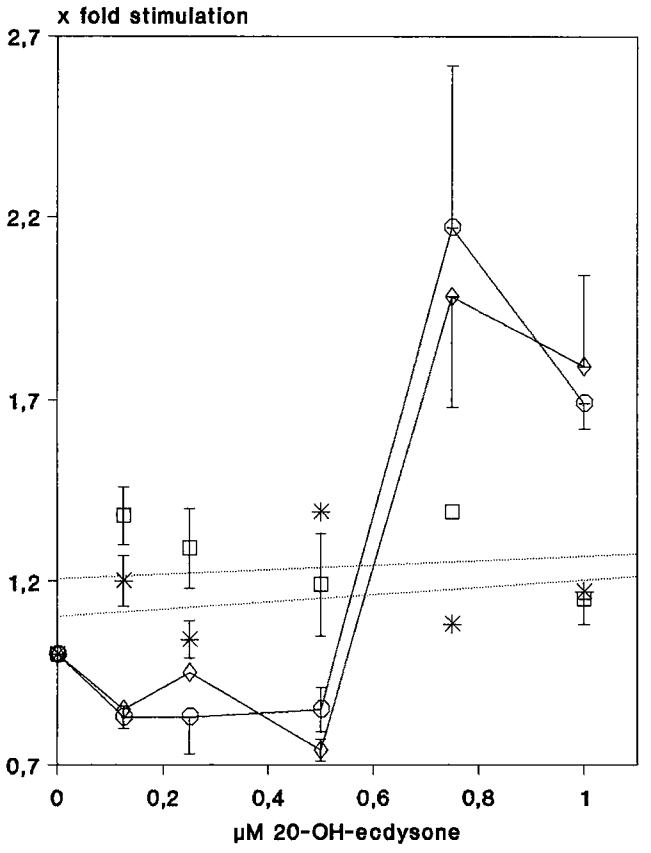

FIG. 6. Dose-response curves for the influence of 20-OH-ecdysone on chitinase activity in wild type and resistant subclones of epithelial *Chironomus tentans* cells. Cells were treated with hormone for 7 d. ◇ = s.0, ○ = s.9 sensitive clones), □ = ec-r.0, ✳ = ec-r.2 (ecdysteroid-resistant subclones). Means ± standard deviations, n = 6. If no standard deviations are given S. D.s were smaller than the symbols and omitted for clarity.

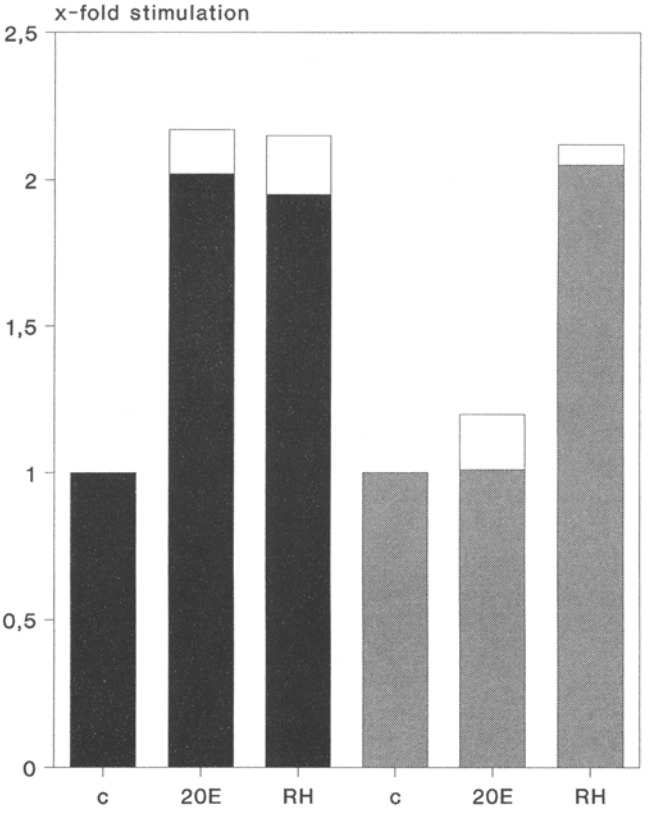

FIG. 7. Influence of 1 µ*M* 20-OH-ecdysone (20E) and 0.1 µ*M* RH 5992 (RH) on chitinase activity in sensitive cells (■; wild type) and a resistant (▨;ec-r.0) subclone of the epithelial cell line from *Chironomus tentans*. Cells were incubated with hormone or agonist for 7 d. Means ± standard deviations (□), n = 3.

both RH 5992 and 20-OH-ecdysone. This is evident according to morphological criteria (data not shown) and also from the inability to induce acetylcholinesterase activity in these subclones (Table 1).

Stability of resistance. In order to discriminate between resistance due to irreversible loss of hormone responsiveness and transient events like receptor down regulation, we propagated resistant clones in the absence of hormone and RH 5992 for at least 3 mo. Renewed incubation with 20-OH-ecdysone or the hormone agonist revealed that the hormonal response remained unchanged as is demonstrated in Table 2 for acetylcholinesterase activity. This means that clones originally resistant only to 20-OH-ecdysone but not to RH 5992 (ec-r.0, ec-r.1, ec-r.2) still do not respond to molting hormone, but are sensitive to the hormone mimic, whereas clones resistant to hormone analogue alone (rh-r.1, rh-r.2, rh-r.3, rh-r.4) or in combination with ecdysteroids (ec-rh.3) remained unresponsive to both. Viability and growth rates of resistant subclones are comparable to wild type cells independent of the presence or absence of hormone or agonist.

DISCUSSION

Several laboratories made efforts to select hormone-resistant cells, mainly from *Drosophila* cell lines (Courgeon, 1972, 1975; Stevens

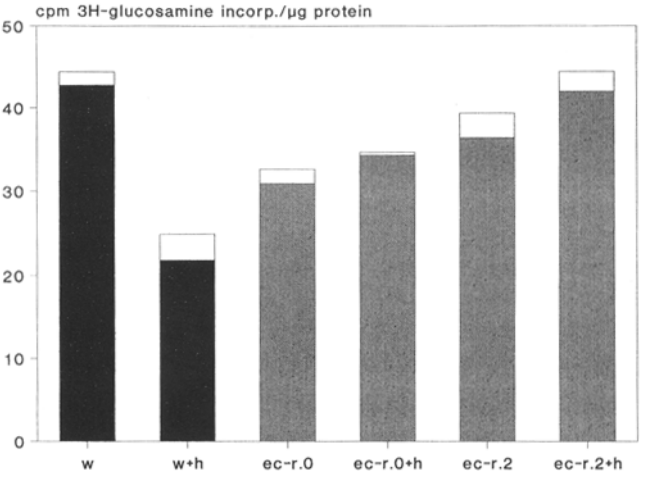

FIG. 8. Chitin synthesis in hormone sensitive cells (■; w = wild type) and resistant subclones (▨; ec-r.0, ec-r.2) of *Chironomus tentans* cells in the presence of 1 µ*M* 20-OH-ecdysone (+h). Incorporation of ^{3}H-glucosamine into chitin was measured after 7 d of incubation without or with 20-OH-ecdysone. Means ± standard deviations (□), n = 3. Values are expressed as percent of the control.

and O'Connor, 1982; Koelle et al., 1991). Generally, these subclones exhibit reduced viability, so they could be maintained in the laboratory only for a limited time period. However, according to L. Cherbas (personal communication), these problems are overcome recently. Koelle et al. (1991) report fluctuations of hormone sensitivity in resistant clones. After several months under continuous presence of molting hormone, resistant sublines respond again to ecdysteroids in an unpredictable and nonreproducible fashion. In some cases, binding studies with radiolabeled ponasterone A (Stevens and O'Connor, 1982) and titration of the mRNA with the corresponding cDNA of the ecdysteroid receptor (Koelle et al., 1991) revealed a considerably reduced ecdysteroid receptor level. In most instances, however, the molecular defect, which leads to hormone resistance, is so far unknown.

Resistant subclones selected from the epithelial cell line from *Chironomus* revealed two classes of hormone resistance: ecdysteroid unresponsive cells, which retained their sensitivity to the hormone mimic RH 5992, and subclones, which are resistant to the molting hormone and its nonsteroidal analogue. In both cases, the lack of sensitivity to the hormonal stimulus is observed for all responses, which are elicited by ecdysteroids in the wild type cells, either involved in the morphogenetic reaction (morphology, acetylcholinesterase activity) or chitin metabolism or thymidine incorporation (data not shown). Membrane-bound enzymes like acetylcholinesterase and chitin synthase, or proteins secreted into the culture medium (chitinase), are equally affected; enzyme induction (acetylcholinesterase, chitinase) as well as inhibition (chitin synthase) are no longer possible in the same resistant subclones. This may indicate that not a certain hormone dependent structural gene is affected, but that the hormone signaling pathway itself is interrupted.

As shown previously, the nonsteroidal hormone agonists RH 5849 and 5992 elicit all the multiple ecdysteroid effects described in this cell line so far. Generally, there is a good correlation between receptor occupancy and biological response in *Chironomus* cells, as shown previously (Spindler-Barth et al., 1991; Quack et al., 1995). Therefore, it seems reasonable that in ecdysteroid resistant, but RH 5992 sensitive, cells, which also show the same dose response curve for the hormone agonist as wild type cells (data not shown), the hormone receptor is functional and all subsequent steps are still intact. Subsequent analysis of hormone metabolism in these clones revealed that ecdysteroid metabolism leading to biological inactive products (Kayser, personal communication) is considerably increased. However, in addition to increased 20-OH-ecdysone metabolism, the receptor concentration is reduced at least in some clones (Rauch, personal communication). Therefore, the hormone response is only partially restored.

Preliminary results show that in some subclones, which are resistant to both ecdysteroids and benzoylhydrazines, ligand binding to the ecdysteroid receptor is affected. Altered receptor expression, defects in receptor-DNA binding, or posttranslational modification of the receptor molecule are also possible causes and are currently under investigation.

The resistant subclones of the *Chironomus* cell line are stable even after propagation in the absence of molting hormone or nonsteroidal agonist. This rules out transient receptor down regulation. The isolation of a number of viable subclones, which exhibit long-term resistance, may be helpful to examine in more detail the mechanism and regulation of the hormone signaling pathway at the molecular and cellular level. This is already demonstrated successfully by L. Cherbas (personal communication), who showed with the help of an ecdysteroid-resistant clone of the Kc cell line from *Drosophila*, that the nonligated ecdysteroid receptor exerts its own specific biological function.

TABLE 1

INFLUENCE OF 1 μ*M* 20-OH-ECDYSONE AND THE NONSTEROIDAL MOLTING HORMONE MIMIC RH 5992 (0.1 μ*M*) ON ACETYLCHOLINESTERASE ACTIVITY IN THE EPITHELIAL CELL LINE FROM *CHIRONOMUS TENTANS* WILD TYPE AND RESISTANT SUBCLONES[a]

Subclone	1 μ*M* 20-OH-ecdysone	0.1 μ*M* RH 5992
Wild type	20.1 ± 8.0	13.8 ± 1.6
rh-r.1	0.8 ± 0.4	1.0 ± 0.6
rh-r.2	0.7 ± 0.1	0.7 ± 0.1
rh-r.3	1.0 ± 0.4	1.5 ± 0.4
rh-r.4	1.2 ± 0.3	1.3 ± 0.3
rh-r.5	0.8 ± 0.2	1.0 ± 0.3
rh-r.6	1.5 ± 0.4	2.3 ± 0.4
ec-rh.0	1.5 ± 0.3	2.0 ± 0.4
ec-rh.2	0.7 ± 0.2	1.0 ± 0.1

[a]Resistant subclones were selected in the permanent presence of RH 5992 alone (rh-r.) or they were selected from 20-OH-ecdysone resistant subclones, which were subsequently kept in the permanent presence of RH 5992 (ec-rh.). Values are expressed as x-fold increase of the corresponding subclone kept hormone-free. n = 4; m ± S.D.

TABLE 2

INFLUENCE OF 1μ*M* 20-OH-ECDYSONE AND THE NONSTEROIDAL MOLTING HORMONE MIMIC RH 5992 (0.1 μ*M*) ON ACETYLCHOLINESTERASE ACTIVITY IN THE EPITHELIAL CELL LINE FROM *CHIRONOMUS TENTANS* WILD TYPE AND RESISTANT SUBCLONES[a]

Subclone	1 μ*M* 20-OH-ecdysone	0.1 μ*M* RH 5992
Wild type	50.0 ± 3.7	12.4 ± 3.1
ec-r.0	1.3 ± 0.3	8.8 ± 2.3
ec-r.1	2.8 ± 1.8	30.0 ± 3.7
ec-r.2	1.6 ± 0.4	5.9 ± 0.7
rh-r.1	2.9 ± 0.3	1.5 ± 0.2
rh-r.3	1.4 ± 0.3	0.9 ± 0.1
rh-r.4	0.8 ± 0.1	0.7 ± 0.1
ec-rh.3	1.2 ± 0.3	1.0 ± 0.2

[a]Resistant subclones selected either in the presence of 20-OH-ecdysone (ec-r), RH 5992 (rh-r) or first with 20-OH-ecdysone followed by RH (ec-rh) were kept free of hormone or hormone mimic for 3 mo and then incubated with 20-OH-ecdysone or RH 5992. Values are expressed as x-fold increase of the corresponding subclone kept hormone-free. n = 4; m ± S.D.

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