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The effects of several ecdysteroids and ecdysteroid agonists on two *Drosophila* imaginal disc cell lines

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Abstract. Two *Drosophila* imaginal disc cell lines, Cl8+ (sensitive to 20-hydroxyecdysone, 20HE) and Cl8R (resistant to 20HE) were exposed to the ecdysteroid agonists RH5849 and RH5992 and the ecdysteroids inokosterone, makisterone A and muristerone A. All compounds tested were found to have similar effects on the cells, comparable to the effects of 20HE, although at different concentrations. Cl8R showed resistance to all compounds, again at varying concentrations. We conclude that it is likely that all the compounds tested use the same receptors as 20HE, but show maximum effectiveness at different concentrations.
Key words. *Drosophila*; cell lines; ecdysteroids; ecdysteroid agonists.

Introduction

The use of *Drosophila* cell lines as research tools for the study of hormone action has become well established in recent years. Various lines have been used to investigate the action of 20-hydroxyecdysone (20HE) in some depth. However, the number of known ecdysteroids is constantly expanding; the Ecdysone Handbook lists over 200 compounds, not all of them 'true ecdysteroids' but including a large number of naturally occurring zoo- and phyto-ecdysteroids [1]. In addition, nonsteroidal ecdysteroid agonists, primarily RH5849 and RH5992, have been added to the list of compounds to be investigated.

In this laboratory we have developed a method to obtain cell lines from the imaginal discs of third instar larvae [2, 3]. A set of clones was derived from a wing disc line; these vary in responsiveness to 20HE, and two of them, Cl8+ and Cl8R, were used in this study. Cl8+ is hormone responsive, while the subline Cl8R was derived from Cl8+ by growing the cells in the presence of increasing concentrations of 20HE until a nonresponsive line was established [4]. This line is presumed to have lost most of its ecdysteroid receptor content; at high concentrations of 20HE a response is seen, so we cannot assume that all receptors are absent. We wished to investigate the effect of some naturally occurring ecdysteroids and the two nonsteroidal ecdysteroid agonists on these two cell lines.

The effects of 20HE on Cl8+ and Cl8R have been previously characterized, and the responses resemble those of a number of other widely-used lines [5–7]. In Cl8+, characteristic morphological changes are observed, cells stop proliferating, and enzyme induction occurs. In the presence of concentrations of 2×10^{-8} M (10 ng ml^{-1}) 20HE and above, some cells produce elongated cell processes and aggregate. Single cells or small clumps produce layers of cuticle which may be tanned. Many cells seem to have more inclusions or vacuoles in the cytoplasm after hormone exposure. Cl8+ also shows β-galactosidase induction in the presence of 20HE, as do a number of other lines [8]. Cl8+ also produces β-galactosidase in the presence of the other ecdysteroids and ecdysteroid agonists used in this study (data not shown). When originally established, Cl8R was resistant to 20HE at a concentration of 150 ng ml^{-1} (approx 3×10^{-7} M).

The responses of Cl8+ and Cl8R to 20HE were used as references to test the effects of a number of other compounds. The nonsteroidal ecdysteroid agonists RH5849 and RH5992, both of which mimic a number of effects of 20HE, were used, in addition to the ecdysteroids inokosterone and makisterone A and the phyto-ecdysteroid muristerone A [1, 6, 9–12]. RH5849 and RH5992 have been found to bind to the same receptors as 20HE, RH5849 with about one-hundredth the potency of 20HE and RH5992 more potent than 20HE by about tenfold [13].

Materials and methods

Cell lines were cultured in modified Shields and Sang's M3 medium supplemented with 2% fly extract and 2.5% FCS [2, 14]. The cells were passaged at intervals of 7–10 days, with no hormone used in routine culture. The cells used in the experiments were at passages 54–58, or 24–28 after cloning, within the usual age range of cells used for experiments in this laboratory.

All hormones were tested at concentrations of 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M with hormone-free controls. RH5849 and RH5992 (gifts from Rohm and Haas) and muristerone A (Simes) were initially dis-

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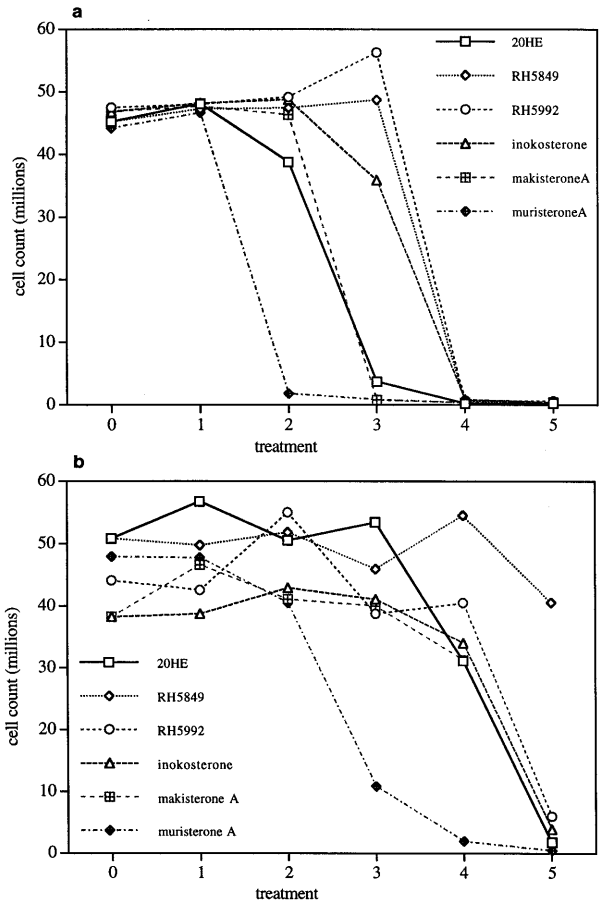

Figure 1. (*a*) Effects of a range of hormones on Cl8+; (*b*) Effects of a range of hormones on C18R. Treatments: 0 = control, 1 = 10^{-9} M, 2 = 10^{-8} M, 3 = 10^{-7} M, 4 = 10^{-6} M, 5 = 10^{-5} M.

solved in ethanol before further dilution with culture medium, while 20HE (Simes), inokosterone (Rohto) and makisterone A (Simes) were dissolved and diluted in culture medium. The final concentration of ethanol in test cultures was less than 1%, a concentration which has been shown to cause no detectable difference in cell growth or morphology in these cell lines (unpublished data).

Three cultures were set up at each concentration for each of the two lines. Initial cell density was 3×10^6 cells per 5 cm dish in 5 ml medium. After 7 days cells were examined, photographed and harvested. The cells were counted using a haemocytometer, to derive a total count of cells in that culture. Data were analysed using one-way analysis of variance with a post-hoc Tukey test (error rate 0.05) for statistically significant differences in cell numbers at different treatments.

Results

The cell counts of cultures harvested after 7 days' growth in the presence of various hormones are represented graphically in figures 1a (Cl8+) and 1b (Cl8R). In Cl8+ cultures, a statistically significant drop in numbers occurred at 10^{-8} M muristerone A , 10^{-7} M 20HE, inokosterone and makisterone A, and at 10^{-6} M RH5849 and RH5992. The groupings of hormones showing each level of effectiveness can be clearly seen in the graph. In Cl8R cultures, the equivalent drop in numbers was seen at 10^{-6} M 20HE and 10^{-5} M inokosterone, makisterone A, and RH5992. In Cl8R treated with RH5849 a significant but less marked decrease also occurred at 10^{-5} M, while muristerone A-treated cultures showed a relatively small but significant decrease at 10^{-8} M, followed by further marked decreases at higher dose levels. Muristerone A stands out from the other compounds as markedly more effective on both cell lines. In most higher dose levels, especially in Cl8+, the cell count indicated fewer cells than the seeding level (3×10^6), due to cell death and lysis. The approximate concentrations of hormones required to cause a 50% drop in cell numbers for each line are shown in table 1.

All hormones tested gave a similar response in that lower treatment levels showed no effect, then a marked drop in cell proliferation occurred before response levelled off in higher doses with only a fraction of those cells originally seeded remaining. In some cases an intermediate level could be seen, usually much closer to one extreme than the other. In a number of cases, a common effect of an increase in cell proliferation at low dose levels was observed (frequent in these cell lines, and also noted elsewhere), but the increases were not statistically significant in these experiments [15].

In Cl8R, resistance to the effects of all hormones was exhibited, with effects similar to those shown by Cl8+ but at higher doses.

The morphological effects of all the hormones were similar, as described above, differing only in the dose level at which they were initiated. The first markedly affected treatment for each hormone is shown in figure 2, together with two control cultures.

Table 1. Approximate concentrations of hormone at which cell numbers were reduced to 50% of control levels (LC_{50}) for the two cell lines.

	20HE	RH5849	RH5992	Inokosterone	Makisterone A	Muristerone A
Cl8+	6×10^{-8}	6×10^{-7}	6×10^{-7}	4×10^{-7}	5×10^{-8}	6×10^{-9}
Cl8R	3×10^{-6}	-	6×10^{-6}	5×10^{-6}	5×10^{-6}	6×10^{-8}
Approximate ratio of LC_{50} doses for Cl8+ and Cl8R	100	-	10	10	100	10

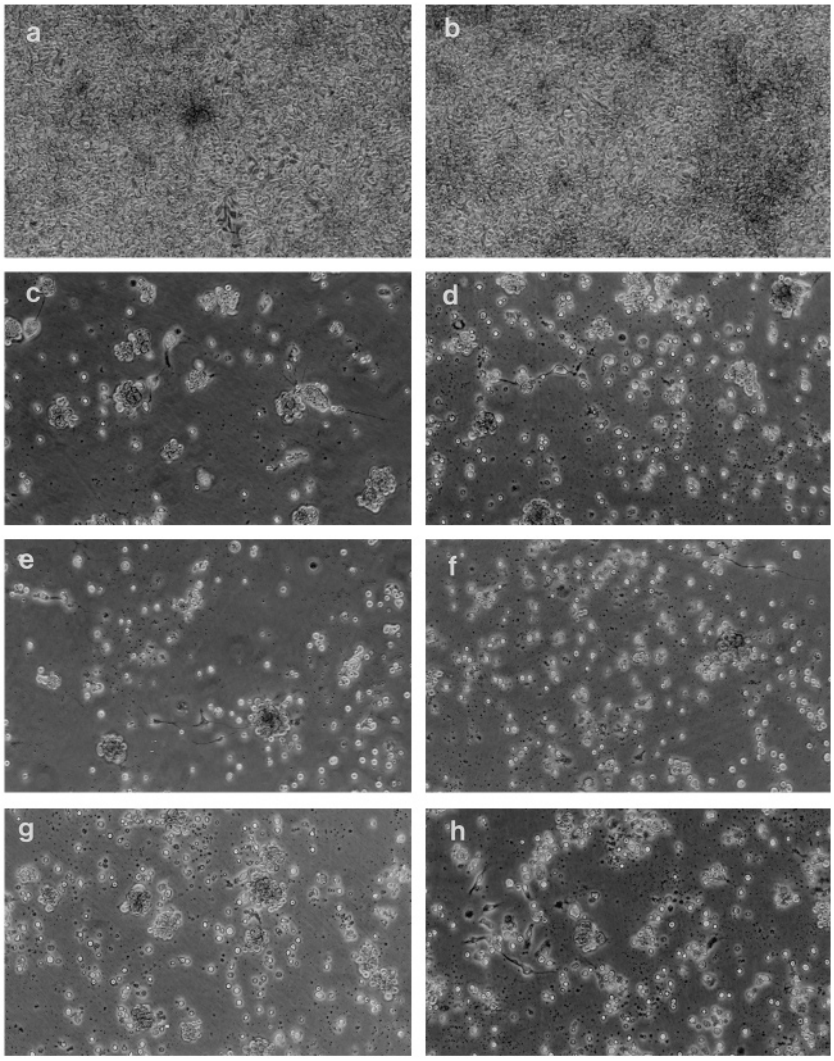

Figure 2. Cells of Cl8+ treated with various hormones. (*a*, *b*) Controls (treatment 0), (*c*) 10^{-7} M 20HE (treatment 3), (*d*) 10^{-6} M RH5849 (treatment 4), (*e*) 10^{-7} M RH5992 (treatment 3), (*f*) 10^{-6} M inokosterone (treatment 4), (*g*) 10^{-7} M makisterone A (treatment 3), (*h*) 10^{-8} M muristerone A (treatment 2).

Discussion

The morphological effect of all the compounds tested in this study were very similar. The cultures shown in figure 2c–h were indistinguishable. The difference between compounds lay in the critical dose level causing a marked response. Muristerone A proved the most potent compound, with an effect between 10^{-9} and 10^{-8} M in Cl8+. Makisterone A and 20HE showed broadly similar effects, with the crucial dose between 10^{-8} and 10^{-7} M. The remaining compounds, RH5849, RH5992 and inokosterone, showed marked effects between 10^{-7} and 10^{-6} M, requiring higher dose levels than 20HE to give the same effect. Levels of hormone about 10–100 times higher were required to stimulate the same response in Cl8R as in Cl8+. In the case of RH5849, the decrease in Cl8R at concentrations ten times those showing a decrease in Cl8+ is not as marked, but still significant. Cl8R treated with makisterone A shows a significant decrease at concentrations 100 times those required to affect Cl8+; the decrease occurring at 10^{-6} M is not statistically significant compared to the control.

Relative effectiveness of different compounds are in broad agreement with previous studies mentioned in the introduction. Moreover, Cl8R has retained its resistance at about the same level recorded in 1992, despite not being routinely cultured in the presence of 20HE for at least ten passages. The cells were stored in liquid nitrogen for about five years, which is not included as part of their age.

As mentioned previously, it has been reported that the ecdysone agonists bind to the same receptors as do 20HE and the other ecdysteroids [10, 13]. The similarity of effects of the different hormones, in terms of morphology, reduction in cell numbers and β-galactosidase induction (data not shown) support this view. The

fact that resistance to one hormone, such as C18R's resistance to 20HE, can confer resistance to the others also offers evidence for common receptor; this has also been noted in Kc cells [9]. Evidence from various studies suggests that the different potencies of the different compounds could be dictated by affinity for receptor, so that level of receptor binding dictates responses [13]. Tests of levels of biological activity RH5849 and RH5992 have shown correlation between activity of the hormone and binding affinity for ecdysteroid receptor (EcR) [16, 17]. It is now known that there is more than one form of ecdysteroid receptor in *Drosophila*, and that ultraspiracle protein (USP) is required for binding in most cases [18, 19]. In imaginal discs isoform EcR-A seems to predominate, with EcR-B1 much more weakly expressed. DHR38 can also form a heterodimer with USP, competing with EcR and disrupting EcR/USP binding to the ecdysone response element [20]. It seems possible seven-up protein may also heterodimerize with EcR, with a repressive rather than inductive effect [21]. Ecdysone response could be modulated by these effects, or by limited concentrations of the various factors. Furthermore, binding affinity of hormone for EcR may be modified by the particular partner in complex with EcR [22].

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1 Lafont R. D. and Wilson I. D. (1996) The Ecdysone Handbook, 2nd edn, Chromatographic Society Surveys, Chromatographic Society, Nottingham
2 Currie D. A., Milner, M. J. and Evans C. W. (1988) The growth and differentiation in vitro of leg and wing imaginal disc cells from *Drosophila melanogaster*. Development **102:** 805–814
3 Milner M. J. (1996) *Drosophila* Cell and Tissue culture. In: Cell and Tissue Culture: Laboratory Procedures, Ch. 24A, Doyle A., Griffiths J. B. and Newell D. G. (eds), John Wiley and Sons, Chichester
4 Peel D. J. and Milner M. J. (1990) The diversity of cell morphology in cloned lines derived from *Drosophila* imaginal discs. Roux's Arch. Devl Biol. **198:** 479–482
5 Peel D. J. and Milner M. J. (1992) The response of *Drosophila* imaginal disc cell lines to ecdysteroids. Roux's Arch. Devl Biol. **202:** 23–35
6 Courgeon A. M. (1972b) Action of insect hormones at the cellular level: morphological changes of a diploid cell line of *Drosophila melanogaster* treated with ecdysone and several analogues *in vitro*. Expl Cell Res. **74:** 327–336
7 Berger E., Ringler R., Alahiotis S. and Frank M. (1978) Ecdysone-induced changes in morphology and protein synthesis in *Drosophila* cell cultures. Devl Biol. **62:** 498–511
8 Best-Belpomme M., Courgeon A. M. and Rambach A. (1978a) β-galactosidase is induced by hormone in *Drosophila melanogaster* cell cultures. Proc. Natl Acad. Sci. USA **75:** 6102–6106
9 Wing K. D. (1988) RH5849, a non-steroidal ecdysone agonist: effects on a *Drosophila* cell line. Science **241:** 467–469
10 Oberlander H., Silhacek D. L. and Porcheron P. (1995) Non-steroidal ecdysone agonists: tools for the study of hormonal action. Arch. Insect Biochem. Physiol. **28:** 209–223
11 Faux A., Horn D. H. S., Middleton E. J., Fales H. M. and Lowe M. E. (1969) Moulting hormones of a crab during ecdysis. Chem. Commun. 175–176
12 Kaplanis J. N., Dutky S. R., Robbins W. E., Thompson M. J., Lindquist E. L., Horn D. H. S. et al. (1975) Makisterone A: a 28-carbon hexahydroxy molting hormone from the embryo of the milkweed bug. Science **190:** 681–682
13 Quack S., Fretz A., Spindler-Barth M. and Spindler K.-D. (1995) Receptor affinities and biological responses of non-steroidal agonists on the epithelial cell line from *Chironomus tentans* (Diptera: Chironomidae). Eur. J. Entomol. **92:** 341–347
14 Cullen C. F. and Milner M. J. (1991) Parameters of growth in primary cultures and cell lines established from *Drosophila* imaginal discs. Tissue and Cell **23:** 29–39
15 Wyss C. (1976) Juvenile hormone analogue counteracts growth stimulation and inhibition by ecdysone in clonal *Drosophila* cell lines. Experientia **32:** 1272–1274
16 Smagghe G., Eelen H., Verschelde E., Richter K. and Degheele D. (1996) Differential effects of non-steroidal ecdysteroid agonists in Coleoptera and Lepidoptera: analysis of evagination and receptor binding in imaginal discs. Insect Biochem. Molec. Biol. **26:** 687–695
17 Wing K. D., Slawecki R. A. and Carlson G. R. (1988) RH5849, a nonsteroidal ecdysone agonist: effects on larval Lepidoptera. Science **241:** 470–472
18 Talbot W. S., Swyryd E. A. and Hogness D. S. (1993) *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. Cell **73:** 1323–1337
19 Yao T.-P., Segraves W. A., Oro A. E., McKeown M. and Evans R. M. (1992) *Drosophila* ultraspiracle modulates ecdysone receptor function via heterodimer formation. Cell **71:** 63–72
20 Sutherland J. D., Kozlova T., Tzertzinis G. and Kafatos F. C. (1995) *Drosophila* hormone receptor 38: a second partner for *Drosophila* USP suggests an unexpected role for nuclear receptors of the nerve growth factor-induced protein B type. Proc. Natl Acad. Sci. USA **92:** 7966–7970
21 Thummel C. S. (1995) From embryogenesis to metamorphosis: the regulation and function of *Drosophila* nuclear receptor superfamily members. Cell **83:** 871–877
22 Yao T.-P., Forman B. M., Jiang Z., Cherbas L., Chen J.-D., McKeown M. et al. (1993) Functional ecdysone receptor is the product of EcR and *Ultraspiracle* genes. Nature **366:** 476–479

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