solve this question, they also support the receptor hypothesis.

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23. The somewhat smaller conductance seen with 5HT could be related to these eggs having been matured in vitro, compared to in vivo maturation for the fertilization series. Biological variation is also probably an important factor because a previous study (*13*) reported a peak conductance of 13 μS during fertilization of *Xenopus* eggs in F1 medium.
24. The series of experiments with M1 (Fig. 2C) was done at a different season than the series of experiments with sperm and 5HT (Fig. 2, A and B); this may account for the approximately 20% smaller initial capacitance values in the M1 series. Another study of the capacitance of the unfertilized *Xenopus* egg gave an even smaller value (55 nF) (*15*), supporting the idea that there may be considerable biological variation among frogs. The shorter duration of the capacitance response may be due to biological variation or may be a real difference between sperm and ACh activation.
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28 March 1988; accepted 10 May 1988

RH 5849, a Nonsteroidal Ecdysone Agonist: Effects on a *Drosophila* Cell Line

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The steroid molting hormone 20-hydroxyecdysone is the physiological inducer of molting and metamorphosis in insects. In ecdysone-sensitive *Drosophila* K_c cells, the insecticide RH 5849 (1,2-dibenzoyl-1-*tert*-butylhydrazine) mimics the action of 20-hydroxyecdysone by causing the formation of processes, an inhibition of cell proliferation, and induction of acetylcholinesterase. RH 5849 also competes with [^{3}H]ponasterone A for high-affinity ecdysone receptor sites from K_c cell extracts. Resistant cell populations selected by growth in the continued presence of either RH 5849 or 20-hydroxyecdysone are insensitive to both compounds and exhibit a decreased titer of measurable ecdysone receptors. Although it is less potent than 20-hydroxyecdysone in both whole-cell and cell-free receptor assays, RH 5849 is the first nonsteroidal ecdysone agonist.

THE TWO NONPEPTIDE HORMONES known to regulate insect metamorphosis and development are the sesquiterpenoid juvenile hormone and the steroid molting hormone 20-hydroxyecdysone (Fig. 1) (*1*). Juvenile hormone, which is responsible for maintenance of a larval or nymphal state in immature molting insects, has been the subject of intensive chemical research and has served as a model for an entire class of structurally diverse molecules that mimic juvenile hormone (*2*). By contrast, although the use of ecdysones as insecticides has been considered (*3*), progress has been hampered by the structural complexity and synthetic inaccessibility of the active steroids for commercial scale field application. In addition, insects have developed powerful mechanisms for catabolizing and clearing ecdysones between molts (*4*). Thus the molting hormones have received little attention from the pesticide industry.

The discovery made over 50 years ago that keto-phenanthrenes can have estrogenic effects spawned both an active area of basic biomedical research and an approach to novel drug development (*5*). However, the absence of such nonsteroidal ecdysone agonists and antagonists has prevented similar advances in the field of insect endocrinol-

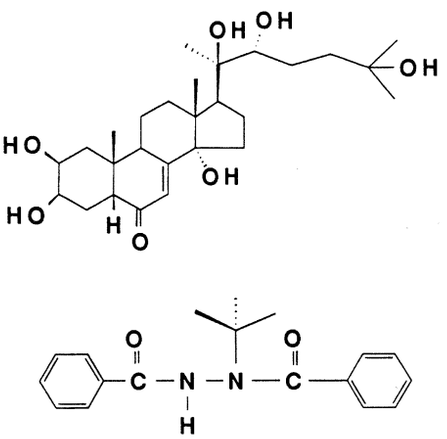

Fig. 1. Structures of (**top**) the invertebrate molting hormone 20-hydroxyecdysone and (**bottom**) RH 5849.

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ogy. I report here that the insecticide RH 5849 [1,2-dibenzoyl-1-*tert*-butylhydrazine (Fig. 1) (*6*)] is a nonsteroidal ecdysone agonist in *Drosophila* K_c cell extracts and whole cells and in larval Lepidoptera (*7*).

The K_c cell line, originally derived from *Drosophila* embryos (*8*), has proved to be an excellent model system for the study of ecdysone action on differentiation and morphogenesis (*9*) and early events in genomic regulation of protein synthesis (*10*). Naïve K_c cells divide every 24 hours and are roughly spherical; however, they differentiate after exposure for 2 days or more to ecdysones by halting their proliferation, clumping tightly, and forming long, branched processes (*11*). RH 5849–treated cells were in all respects indistinguishable morphologically from 20-hydroxyecdysone–treated cells (Fig. 2). However, the hormone concentration for 50% of maximal response or EC_{50} (0.035 μ*M*) was about 1/137 of that for RH 5849 (EC_{50} = 4.8 μ*M*) as measured by process elaboration and inhibition of proliferation (Fig. 3). These characteristic responses have heretofore been restricted to active ecdysones and cannot be induced by cyclic nucleotides, dimethyl sulfoxide, various mammalian growth factors, or serum deprivation (*11*).

In addition, both 20-hydroxyecdysone (EC_{50} = 0.007 μ*M*) and RH 5849 (EC_{50} = 1.05 μ*M*) cause an increase in the specific activity of acetylcholinesterase (Fig. 4), also a response previously restricted to the ecdysones (*12*). 20-Hydroxyecdysone is 150 times as potent in this assay as RH 5849. The slopes of the dose-response curves for process elaboration, cell density, and acetylcholinesterase induction are roughly parallel for both compounds, arguing for the action of both the steroid and RH 5849 at the same receptor but with different affinities.

Figure 5A shows a Scatchard plot of [^{3}H]ponasterone A (*13*) binding to K_c cell cytosol extracts in the presence of RH 5849; increasing concentrations of RH 5849 lead to an increase in the equilibrium dissociation constant (K_d), whereas the concentration of the maximum number of ligand binding sites (B_{max}) remains the same. These binding thermodynamics indicate a competitive mode of inhibition of [^{3}H]ponasterone A binding and imply that ponasterone A and RH 5849 share a common binding domain in the receptor. The experimentally determined value of K_d from the binding isotherm in the absence of RH 5849 (0.29 n*M*) is lower than a value re-

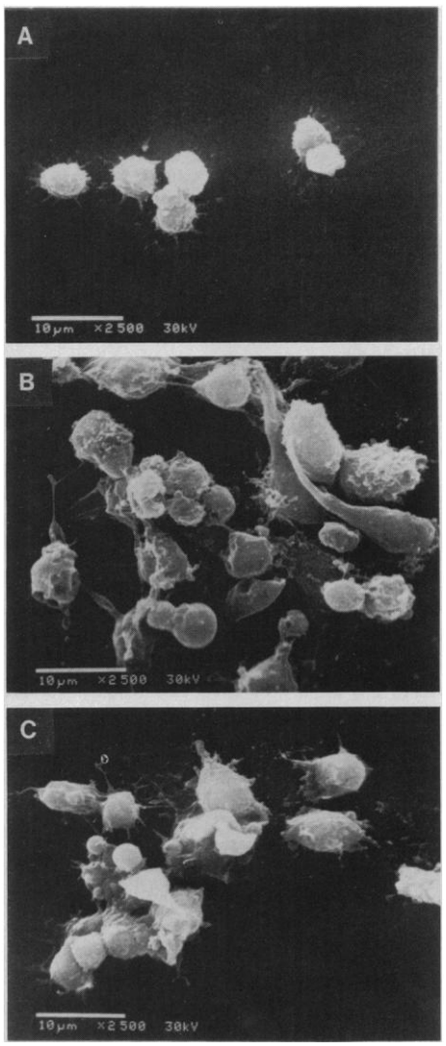

Fig. 2. Scanning electron micrographs of *Drosophila* K_c cells after treatment with (**A**) DMSO carrier (**B**) 1×10^{-6}*M* 20-hydroxyecdysone and (**C**) 1×10^{-4}*M* RH 5849. Note that the cells in (B) and (C) have become irregularly shaped, have clumped, and have formed long, spindly processes. Cells were incubated with the compound for 48 hours in M3 medium (*18*) containing 12.5% fetal bovine serum (Gibco). The cells were then allowed to stick for 30 min to glass slides coated with polylysine (Sigma, molecular weight >300,000). After being fixed in 2.5% glutaraldehyde (Sigma) in 0.1*M* sodium cacodylate (*p*H 7.4) buffer for 30 min, they were fixed for 60 min in 1% osmium tetroxide in cacodylate buffer. The samples were dehydrated through a 30 to 100% ethanol series (10 min per wash), incubated in 50 and then 100% amyl acetate for 10 min each, and dried in a Denton DCP-1 Critical Point Drying Apparatus. They were then gold-coated in a Denton Desk II Sputter Coater before examination with an Amray 1200C Scanning Electron Microscope. All cells used in this study were from A3A11 clone provided by P. Cherbas.

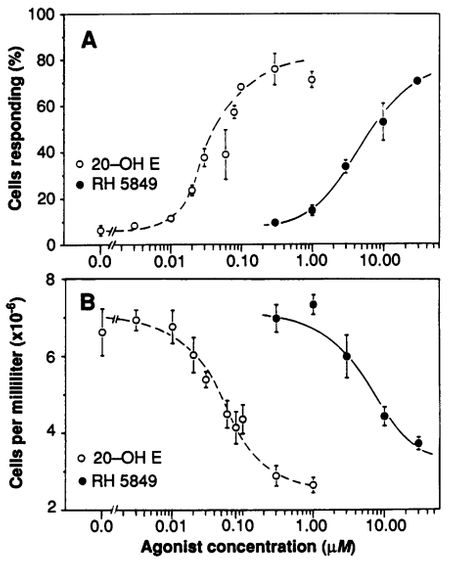

Fig. 3. Both 20-hydroxyecdysone (20-OH E) and RH 5849 induce process elaboration (**A**) and inhibition of cell proliferation (**B**) in K_c cells in a dose-dependent manner. Initial cell density was 2×10^6 cells per milliliter; after incubation of 1-ml aliquots of the cells with the compound for 48 hours the cells were triturated gently. The percentage of cells responding [with processes longer than approximately 17 μm (*11*)] and total cell density were determined by hemocytometer counting, relative to ethanol (1 μl)-treated controls. Determinations were in triplicate for each dose. Data are means ± SD. For 20-hydroxyecdysone EC_{50} was 0.035 μ*M*, whereas for RH 5849, EC_{50} was 4.8 μ*M*. This was a 148-fold difference [±35 (SD)] for three separate determinations.

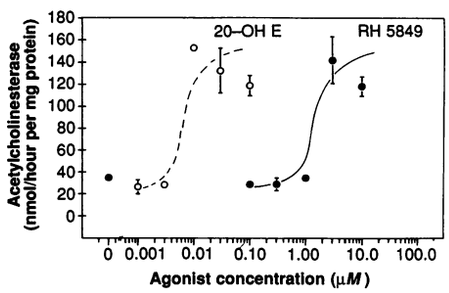

Fig. 4. Induction of acetylcholinesterase by varying concentrations of 20-hydroxyecdysone and RH 5849. K_c cells at an initial density of 3×10^6 cells per milliliter (10 ml per sample) were incubated with the agonist for 3 days. After harvesting by centrifugation at 3000*g*, the cell pellets were washed with Robb's *Drosophila* saline (*19*) (twice with 5 ml) at 4°C, then lysed by sonication in 1 ml of 0.1*M* phosphate buffer (*p*H 7.4) that contained 0.5*M* NaCl, 0.25*M* EDTA, and 0.5% Triton X-100 (*12*). After centrifugation at 16,000*g* the supernatants were assayed for acetylcholinesterase activity by an adaptation of the method of Ellman *et al.* (*20*), by subtracting any 412-nm absorbance observed in incubations in the presence of 2×10^{-5}*M* eserine sulfate. Thus reaction mixtures contained 50 μl of enzyme extract, 20 μl eserine (blank tubes only), and 1.0 ml of 0.5 m*M* acetylthiocholine, 0.3 m*M* dithiobisnitrobenzoic acid in 0.1*M* phosphate buffer (*p*H 7.1). Incubations were at 25°C for 2 hours; reactions in active tubes were terminated by addition of eserine. Absorbance at 412 nm was then measured. Protein concentration in extracts was measured by the Bio-Rad method (*15*). Data are means of triplicate cell incubations plus or minus the standard deviation. After subtraction for eserine-sensitive acetylcholinesterase activity in nontreated cells (30 nmol/hour per milligram of protein) the values for EC_{50} for 20-hydroxyecdysone and RH 5849 were determined to be 0.007 and 1.05 μ*M*, respectively, a 95-fold difference [±27 (SD)] for three separate determinations.

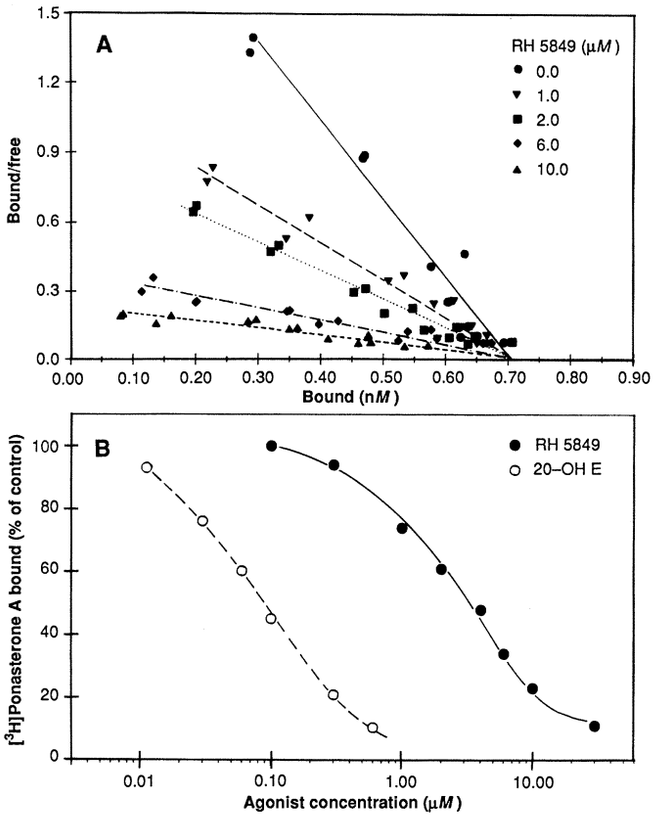

Fig. 5. (**A**) Scatchard plot of [^{3}H]ponasterone A binding to K_c cell cytosolic receptor extracts (*21*) in the presence of differing concentrations of RH 5849. RH 5849 was added in 1 μl of DMSO to 50 μl of radioligand in 10 m*M* tris buffer (*p*H 7.2) and 50 μl of undiluted cytosol extract. After incubation overnight at 0.5°C bound label was separated from free by addition of 300 μl 1% HCl-washed charcoal (Sigma) and 0.1% Dextran T70 (Pharmacia) in tris buffer, centrifugation (13,000*g*, 3 min, 4°C), and determination of radioactivity by liquid scintillation spectrometry of a 300-μl aliquot of the supernatant in 15 ml of Hydrofluor (National Diagnostics). Points represent single tubes; all data are corrected for nonspecific binding (defined as radioactivity bound in the presence of 1×10^{-5}*M* unlabeled 20-hydroxyecdysone). For incubations in the absence of RH 5849, K_d = 0.30 ± 0.02 n*M* and B_{max} = 0.71 ± 0.02 n*M* (mean ± SD, for three separate determinations). (**B**) Relative ability of 20-hydroxyecdysone and RH 5849 to displace [^{3}H]ponasterone A from cytosolic receptor extracts. Conditions were as in (A) except that only 0.5 n*M* ponasterone A was used. Points are means of duplicate determinations. For 20-hydroxyecdysone EC_{50} = 0.1 ± 0.01; for RH 5849 EC_{50} = 3.03 ± 0.59 μ*M* (mean ± SD, for five separate determinations). Control binding was 10 fmol of [^{3}H]ponasterone A bound per milligram of cytosol protein.

ported in the literature (K_d = 3.4 n*M*) (*14*) and seems to be due to use of a different batch of radioligand. The observed B_{max} (0.71 n*M*) is consistent with that reported previously (B_{max} = 1.4 n*M*) (*14*). 20-Hydroxyecdysone (EC_{50} = 0.1 μ*M*) is 30 times more potent than RH 5849 (EC_{50} = 3.0 μ*M*) at displacing 0.5 n*M* [^{3}H]ponasterone from its receptor (Fig. 5B).

When K_c cells are incubated for 4 weeks in either 1×10^{-6}*M* 20-hydroxyecdysone or 1×10^{-4}*M* RH 5849, the surviving cells do not respond to either compound by elaborating processes or slowing their proliferation. Both of these resistant populations also show a dramatically reduced capacity to bind [^{3}H]ponasterone A relative to untreated cells (*15*). This cross-resistance is compelling evidence that 20-hydroxyecdysone and RH 5849 act through the ecdysone receptor.

The benzoylphenylurea insect growth regulators Dimilin and CGA-112913 (*16*) at saturating concentrations failed to produce ecdysone-like responses in whole K_c cells and did not displace [^{3}H]ponasterone A from cytosolic receptor extracts. Thus, at the biochemical and cellular levels RH 5849 is distinct in its actions from the benzoylphenylureas.

The ecdysone receptors of *Drosophila* cells and tissues have become the focus of increasing research interest and seem to be representative models for other steroid hormone receptors (*17*). My data indicate that the insecticide RH 5849 and its analogs could prove to be useful tools with which to study ecdysone action. RH 5849 and its analogs are more synthetically accessible than the steroids, and thus could be used to "map" the receptors, or to provide affinity ligands for purification. In tissues and whole organisms, these compounds could aid in the in vivo localization of receptors, and possibly in classification of ecdysone receptors in invertebrate phyla based on binding to selected agonists.

Note added in proof: The kinetic off-rate constant k_d for the binding of [^{3}H]ponasterone A to the receptor is unaffected by the presence of 3 μ*M* RH 5849, while the on-rate k_a is lowered. These data are consistent with ponasterone A and RH 5849 binding to a common as opposed to an allosteric receptor site.

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27 January 1988; accepted 19 May 1988