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Inhibitors of Eicosanoid Biosynthesis and Their Effect upon *Bacillus thuringiensis* δ-Endotoxin Response in Cultured Insect Cells and Developing Larvae

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Abstract. Twelve inhibitors of eicosanoid biosynthesis were examined for their ability to affect the response of insect cells in vitro and developing larvae to δ-endotoxin from *Bacillus thuringiensis*. The response of cultured insect cells from *Manduca sexta*, *Choristoneura fumiferana*, and *Plodia interpunctella* to CryIA(c) and CryIC protein from *Bacillus thuringiensis* was measured while exposed to various concentrations of specific cyclooxygenase and/or lipoxygenase inhibitors. Five of the inhibitors (curcumin, baicalein, nordihydroguaiaretic acid, indomethacin, and eicosatetraynoic acid) were toxic to the cells at high concentrations (>20 μM). Surprisingly, the same inhibitors had no significant effect upon normal larval development, except for nordihydroguaiaretic acid. No true, consistent difference was detected with either lipoxygenase or cyclooxygenase inhibitors for cells or larvae treated with δ-endotoxin. However, the δ-endotoxin response of insect cells in vitro and developing larvae in the presence of nordihydroguaiaretic acid was strong evidence of an involvement with P_{450} cytochromes in the *B. thuringiensis* toxic response.

Bacillus thuringiensis (Bt) produces a proteinaceous inclusion body during the normal process of sporulation that is insecticidal to various lepidopteran, dipteran, and coleopteran larvae [12]. The activated crystal protein functions at the site of brush border epithelial cells lining the midgut, binding first to specific receptors on the midgut membrane surface [11, 26], and then acting to disturb the functional stability of the membrane through the induction of pore formation and eventual colloid osmotic lysis [16]. Specific biochemical events occurring in the membrane after receptor binding are not understood. However, it is likely that some effect on the membrane phospholipids is necessary prior to pore formation and osmotically induced lysis of the membrane epithelial cells. In fact, Thomas and Ellar [24, 25] described phospholipid involvement in Bt subsp. *israelensis* toxicity toward cultured insect cells. More recently, Haider and Ellar [10] found that crystal protein from three subspecies of Bt caused morphological changes in the lipid bilayer of multilamellar liposomes. Also, a membrane phosphatase purified from midgut epithelial cells of *Heliothis virescens* was inhibited by δ-endotoxin from Bt subsp. *kurstaki* [6]. Plainly, strong evidence exists to suggest that Bt toxins interact through membrane phospholipids to facilitate the toxic response elicited from a variety of lepidopteran and possibly other types of larvae.

Membrane phospholipids serve as reservoirs for polyunsaturated fatty acids that can lead to a diverse group of compounds derived from arachidonic acid. This group of compounds, known collectively as 'eicosanoids', are produced via the cyclooxygenase or lipoxygenase-catalyzed metabolic pathways. Arachidonic acid, released from phospholipids by the action of phospholipases, is enzymatically converted either by a cyclooxygenase to prostaglandin precursors, by a lipoxygenase to prostaglandins, leukotrienes, and related products, or by a cytochrome P_{450} pathway to hydroxyeicosatetraenoic acids and related products. In many mammals and amphibians, eicosanoids play a major role in the regulation of ion and water transport [3, 4]. In invertebrates, eicosanoids are also involved in osmoregulation, controlling the epithelial ion flux

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including sodium and potassium ion flow [9, 21], and are responsible for regulating fluid secretion in the Malpighian tubules of the mosquito *Aedes aegypti* [20]. The same compounds have also been implicated in the immune response of certain insect larvae to bacterial infections [18, 23]. Although insect cell lines do not reflect a fatty acid composition identical with the composition of their culture media, they do appear to possess functional lipid and fatty acid metabolisms [22]. This would suggest that the cell lines are able to regulate their fatty acid composition in a manner analogous to or representative of the original source tissue.

In this report, we examined the effect of specific inhibitors of eicosanoid biosynthesis on the toxic response of δ-endotoxin toward cultured insect cells and developing Indianmeal moth (IMM) larvae. Unusually high cellular toxicities were found for five of the inhibitors; however, the equivalent concentrations were not as toxic for developing larvae. The toxic inhibitor effects toward the cultured cells made it impossible to unequivocally establish any direct effects of eicosanoid inhibitors upon δ-endotoxin activity. However, in developing larvae and in cultured cells, nordihydroguaiaretic acid (NDGA) consistently diminished δ-endotoxin toxicity.

Materials and Methods

Bacterial protein toxin. CryIA(c) protein was purified from crystals of Bt subsp. *kurstaki* HD-73. The bacterial strain was grown on glucose–yeast extract–salts (GYS) medium [28] including sodium citrate (0.1%) for 48 h at 30°C. Spores, crystals, and cellular debris were harvested by centrifugation, and the pellet was washed extensively with 20 mM Tris-hydroxymethyl-aminomethane (Tris), 50 mM KCl, pH 8.5. Crystals were separated from spores and other contaminants by density gradient centrifugation by use of sodium bromide [2]. After extensive washing to remove residual sodium bromide, the purified crystals were dissolved in 0.0135 *N* NaOH for 2 h at a concentration of 2–4 mg/ml. The CryIA(c) protoxin was activated by the addition of 20 µg chymotrypsin (Sigma Chemical Co., St. Louis, Missouri) per mg soluble protoxin and incubated for 2 h at 37°C. The activated CryIA(c) protein was applied to a DEAE Bio-Gel A column (BioRad, Hercules, California) and was eluted with a 0.05–0.3 M gradient of KCl in 20 mM Tris, pH 8.5. Fractions were assayed for activity and the active fractions pooled, dispensed in microcentrifuge tubes at 100 µg/tube, and lyophilized. CryIC protein was derived from Bt subsp. *entomocidus* HD-110 and was kindly provided by Plant Genetic Systems (Ghent, Belgium). It was a recombinant protein extracted and purified from transformed *Escherichia coli* [27]. Protein concentrations for all the preparations used in this work were determined with the BCA Protein Assay Reagent (Pierce, Rockford, Illinois).

Insect cell lines. Cultured insect cell lines from *Manduca sexta* L. (MRRL-CHE-20), *Choristoneura fumiferana* Clemens (IPRI-CF-1), and *Plodia interpunctella* Hübner (KSU-PI5.3) were subcultured as previously described [13].

Eicosanoid biosynthesis inhibitors. All of the compounds tested were specific inhibitors of various pathways leading to eicosanoid biosynthesis. They were obtained from BioMol Laboratories (Plymouth Meeting, Pennsylvania) and included: carboxyheptylimidazole, indomethacin, and naproxen (cyclooxygenase inhibitors); anthralin, baicalein, caffeic acid, esculetin, and nordihydroguaiaretic acid (NDGA; lipoxygenase inhibitors); curcumin, phenidone, and eicosatetraynoic acid (ETYA; cyclooxygenase and lipoxygenase inhibitors); and dexamethasone (phospholipase A_2 inhibitor) (Fig. 1). All were initially dissolved at 1 mg/ml in acetone. Each inhibitor stock solution was subsequently diluted in 3-(N-morpholino)propanesulfonic acid (MOPS) buffered saline solution, pH 7.0, to achieve the desired working concentration (1, 2.5, 5, 10, and 25 µg/ml).

ATP bioassay procedure. The effect of Bt toxin proteins upon cultured insect cells was measured as previously reported by the luciferin-luciferase enzyme technique [14]. The technique measured the residual ATP in surviving cells following treatment with δ-endotoxin protein and was measured with a Lumac Biocounter (Model M2010, Integrated Biosolutions, Monmouth Junction, New Jersey). Toxicity was expressed as an inverse function of the ATP concentration in treated cells compared with untreated control cell preparations. Once the toxic effect of a toxin upon the tissue cells was established, the presence of any additional compound was evaluated. Cellular ATP levels for cells treated with specific eicosanoid inhibitors in the presence of endotoxin protein were compared with cellular ATP levels of toxin-treated cells in the absence of inhibitor. Various concentrations of inhibitor as well as two different concentrations of toxin were used in order to work at the greatest level of cell sensitivity to toxin–inhibitor interactions.

Larval bioassay. Larvae of *Plodia interpunctella* Hübner were bioassayed for inhibitor effects by an apple bioassay technique that treats individual larvae in separate compartments with the toxicant dose applied directly to a small apple cube [15]. The apple cube serves as the only immediate food source; thus, the larva is forced to consume the measured dose before additional food is provided. A minimum of 16 larvae/dose covering at least eight concentrations (ranging from 0.1 to 80.0 µg Bt toxin), with inhibitor applications as specified, were treated in duplicate in compartmentalized bioassay trays (C-D International, Pitman, New Jersey) that accommodated 128 larvae at a time. The condition of the larvae was assessed at 3- to 4-day intervals, and cracked wheat diet [15] was added when the dosed apple cube was completely consumed. Mortality was calculated by use of the log-probit estimation of the LC_{50} for each toxin by the method of Finney [8].

Results

Appropriate controls were run to determine whether the luciferin-luciferase detection method for cellular ATP was sensitive to the inhibitors used in this study. Although some inhibition of luciferin-luciferase detection of ATP by the inhibitors was detected, the level of interference ranged from 5 and 20%, well within acceptable limits for this study. Likewise, the effect of 2–5 µl acetone upon ATP detection in cultured insect cells was also small, ranging from 0 to 3.9% inhibition of the total ATP response (data not shown). Because the cell lines used in this work are known to respond optimally to specific types of Bt toxin protein [13], it

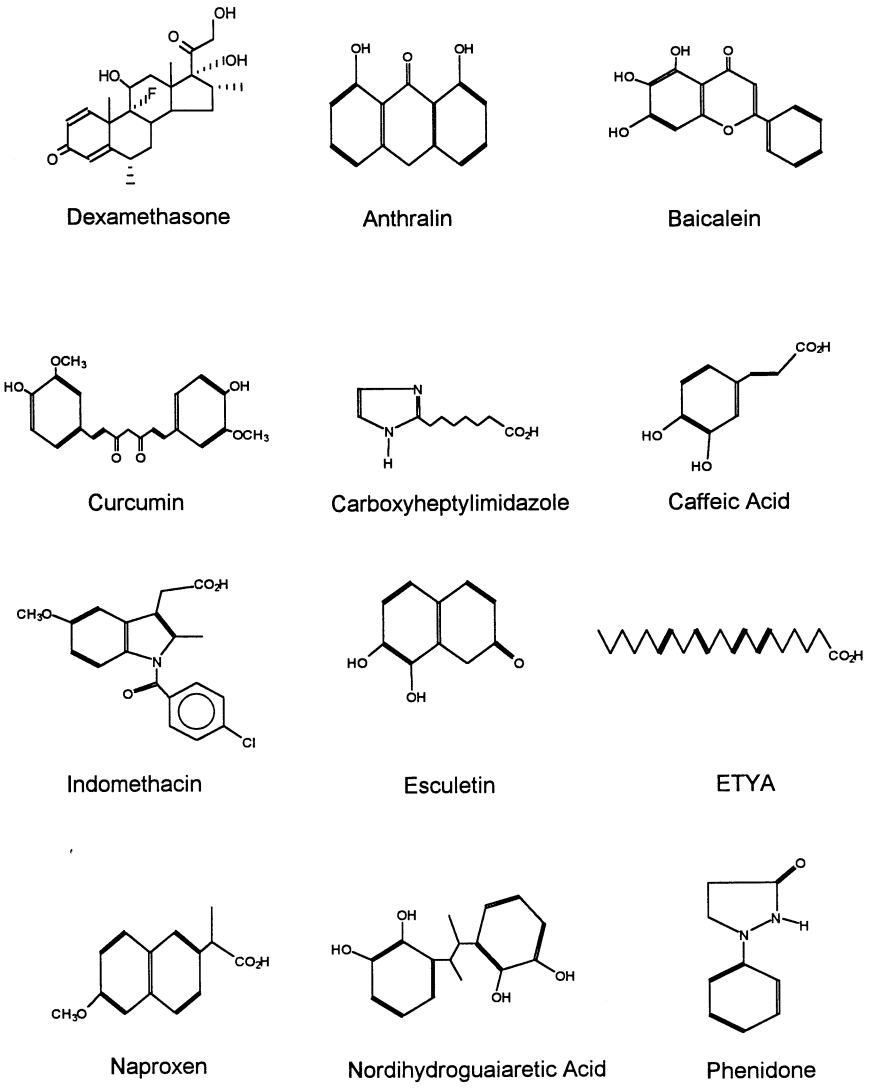

Fig. 1. Chemical structure representations for the 12 eicosanoid inhibitors used in this study.

was necessary to use two particular toxin types. CryIA(c) protein from crystals of Bt subsp. *kurstaki* HD-73 was used for *M. sexta* and *C. fumiferana* cells, and CryIC protein from Bt subsp. *entomocidus* was used for *P. interpunctella* cells.

The inhibitors varied dramatically in their individual effects upon each of the insect cell lines. Actual physiological inhibitor concentrations tested ranged from 3 to 150 μM among the various compounds in solution. Seven of the twelve inhibitors had little or no effect (0–15% loss in viability) at the highest concentration tested (approx. 70–150 μM, depending upon inhibitor). These seven included phenidone, carboxyheptyl imidazole, esculetin, naproxen, caffeic acid, dexamethasone, and indomethacin. The remaining five inhibitors—curcumin, baicalien, ETYA, anthralin, and NDGA—were seriously toxic toward all three cell lines (Fig. 2). The in vitro cellular toxicity for these five compounds as a function of inhibitor concentration for the three species tested was >20% at levels of 5 μg/ml (15–20 μM) and higher. There was no significant difference in the response at each inhibitor concentration for any of the three cell lines, nor was there any difference attributable to the two cry proteins used to elicit a toxic response with each of the three cell lines. Consequently, the individual data from all three cell lines was grouped together and treated as one culture in response to a single toxin.

None of the seven inhibitors that were nontoxic towards the insect cells significantly affected the δ-endotoxin response at the highest dose tested (25 μg/ml). Of the five remaining inhibitors, all caused minor disruption of the Bt-toxic response, but it was not obvious whether the effect was caused by the high

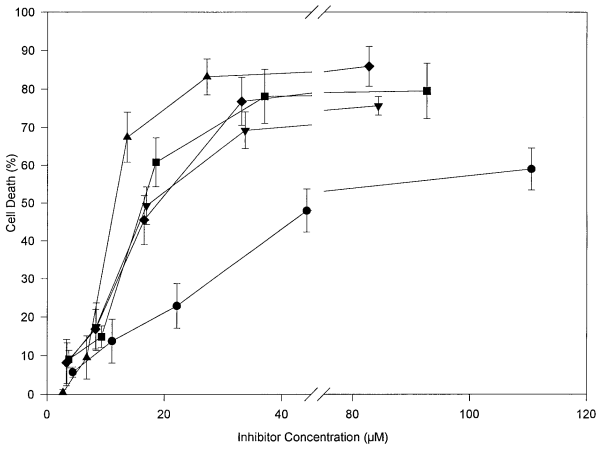

Fig. 2. Cellular inhibition by specific inhibitors of eicosanoid pathways. Data represent combined results from three separate cell lines (*Choristoneura fumiferana*, *Manduca sexta*, and *Plodia interpunctella*). Error bars represent standard deviation. Anthralin (●), baicalein (■), curcumin (▲), ETYA (▼), and NDGA (◆).

Table 1. Effect of eicosanoid inhibitors on *B. thuringiensis* toxicity toward cultured insect cells in vitro

Inhibitor	Type	Conc. (μM)	Bt-Mediated cell response[a]	SD[b]
Dexamethasone	Phospholipase A_2	63.7	1.03	0.16
Anthralin	Lipoxygenase	110.5	0.99	0.25
Baicalein	Lipoxygenase	92.6	1.42	0.50
Caffeic acid	Lipoxygenase	138.7	1.12	0.16
Esculetin	Lipoxygenase	140.0	1.05	0.21
NDGA	Lipoxygenase	82.7	1.51	0.47
Carboxyhepty-limidazole	Cyclooxygenase	118.9	1.11	0.20
Indomethacin	Cyclooxygenase	69.9	0.89	0.13
Naproxen	Cyclooxygenase	108.6	1.06	0.12
Curcumin	Dual (L + C)	68.0	1.37	0.48
ETYA	Dual (L + C)	84.3	1.07	0.44
Phenidone	Dual (L + C)	149.5	1.07	0.14

[a] Ratio of % cell survival in the presence of inhibitor and the LD_{50} dose of *B. thuringiensis* toxin to the % survival in an uninhibited LD_{50} determination.
[b] Standard deviation.

background toxicity of the inhibitors or by their influence upon Bt toxicity. In Table 1, all 12 inhibitors are listed by category according to the type of eicosanoid inhibition in which they are known to interfere. Only three (NDGA, baicalein, and curcumin) inhibited the cellular response to δ-endotoxin, but each compound possessed a high background toxicity for the cells.

Because of the severe toxicity toward cultured insect cells by some of the inhibitors at high concentrations, it was important to determine if these inhibitors were equally as toxic for developing IMM larvae. A series of bioassays were conducted to determine any detrimental effects caused by each of the inhibitors upon survivorship, as judged by adult emergence. Inhibitor concentrations were the equivalent of those used throughout the investigation, and larvae were forced to consume the stated amount of inhibitor before any more food was given [15]. Except for approximately 37.5% toxicity for NDGA (82.7 μM), the rest of the inhibitors were relatively nontoxic towards developing IMM larvae (Table 2). Adult moths emerged from each treatment with no visible impairments and within the normal time interval for development.

Table 2. Effect of eicosanoid inhibitors on *B. thuringiensis* toxicity toward larvae of the Indianmeal moth (*P. interpunctella*)

Inhibitor	Conc. (μM)	*B. thuringiensis*-mediated larval response			
		No.[a]	Bkg[b]	LD_{50}[c]	95% FL[d]
None	—	624	4.69	0.27	0.21–0.34
Baicalein	92.6	524	4.69	0.36	0.25–0.52
NDGA	87.7	524	37.5	0.75	0.59–0.94
Naproxen	108.6	524	6.25	0.37	0.27–0.53
Indomethacin	69.9	524	6.25	0.29	0.21–0.38
ETYA	84.3	524	12.5	0.42	0.31–0.58
Curcumen	68.0	524	12.5	0.31	0.22–0.43

[a] Number of larvae tested.
[b] Inhibitor toxicity in absence of δ-endotoxin.
[c] 50% of the lethal dose, in μg protein/larva.
[d] 95% fiducial limits.

Since the various eicosanoid inhibitors were in general nontoxic toward developing IMM larvae, we wanted to determine if δ-endotoxin larval toxicity would be affected by eicosanoid treatments. The same inhibitors that had shown some alteration of Bt toxicity with cultured insect cells were also used here with intact larvae. Those inhibitors included baicalein, NDGA, naproxen, indomethacin, ETYA, and curcumin. With the exception of some relatively minor and barely significant ($p > 0.5$) effects by specific inhibitors, most did not alter the δ-endotoxin toxicity toward IMM larvae (Table 2). However, NDGA diminished CryIA(c) toxicity more than threefold, while δ-endotoxin toxicity in the presence of each of the other inhibitors varied only within 95% fiducial limits.

Discussion

Three distinct steps are thought to occur in the association of δ-endotoxin with the epithelial membrane of susceptible insects [7]. They are: binding to appropriate receptors, intercalation or partitioning into

the target membrane, and formation of ion channels. Each of these steps involves biochemical processes that may include membrane-bound lipids and the function they play in membrane structure and integrity. Various eicosanoids and phenols can affect insect susceptibility to other pathogens [5, 17, 18, 23]. Some eicosanoids can cause cytoprotective effects in mammalian organs, but these effects are not fully understood in any of the tissues for which documentation is available [19].

Clearly, the potential for interactions between compounds that affect membrane lipid composition and the mode of action of Bt is great. The work of others suggests that membrane phospholipids are clearly involved at the site of midgut disruption. Five of the 12 inhibitors tested had significant background inhibitor toxicity toward the insect cells at high inhibitor concentrations, which made interpretation of the results difficult or impossible. Ideally, we would have expected consistent results with several inhibitors of the same class. Larval development times, pupation, and adult emergence in the presence or absence of the five eicosanoid inhibitors at the stated concentrations were very similar, and the small differences were statistically insignificant. At high concentrations, however, NDGA, a 5- and 12-lipoxygenase inhibitor, was toxic to the larvae, but also had diminished δ-endotoxin toxicity. These data corroborate similar results found with cells in vitro. Baicalein also inhibited δ-endotoxin toxicity in cultured cells, but was ineffective in developing larvae. Esculetin, a 12-lipoxygenase inhibitor, was ineffective towards both cells and developing larvae. NDGA is also known to inhibit microsomal cytochrome P_{450}-mediated monooxygenase activity [1]. This fact, coupled with the inactivity of esculetin, is a positive indicator of the involvement of the P_{450} cytochromes in the cellular and larval response to δ-endotoxin. Work is proceeding to determine the fatty acid composition of cultured cells and whether cultured cells do, in fact, synthesize eicosanoids. These results should enable us to more effectively determine the effects of inhibitors on eicosanoid biosynthesis in cultured insect cells.

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