Biochemical Pharmacology, Vol. 51, pp. 447–454, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
SSDI 0006-2952(95)02200-7

Deltamethrin-Induced Thymus Atrophy in Male Balb/c Mice

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ABSTRACT. The action of deltamethrin, a potent type II synthetic pyrethroid insecticide, on the thymus of the Balb/c mouse was studied *in vivo* and *in vitro*. We found that deltamethrin produced atrophy in the thymus in a dose- and time-dependent fashion. The lowest effective dose was found to be 6 mg/kg, 24 hr after a single intraperitoneal treatment. Treated animals did not recover during the time-course of the experiment (35 days after treatment); however, deltamethrin did not affect the body weight of the treated animals during the course of the study. To determine if deltamethrin-induced $[Ca^{2+}]_i$ signaling could lead to thymic atrophy via programmed cell death, mice were treated with 25 mg deltamethrin/kg for 24 hr or the isolated thymocyte suspension was treated with 50 μM deltamethrin. A significant stimulation of inositol 1,4,5-triphosphate (IP^3) and inositol 1,4-diphosphate (IP_2) production was found after 24 hr of deltamethrin-1R (active isomer) treatment. An inactive stereoisomer of deltamethrin (i.e. 1S) did not cause a significant rise in the production of IP_3 and IP_2. In addition, deltamethrin-1R induced a transient increase of $[Ca^{2+}]_i$ mobilization in the thymocyte suspension after 10 min of *in vitro* treatment, and substantially reduced the rate of calcium-calmodulin (Ca/CaM)-dependent protein dephosphorylation in *in vivo* treated animals (25 mg deltamethrin/kg for 24 hr). The *in vivo* effects of deltamethrin treatment demonstrated induction of DNA fragmentation and cell death in thymocytes. Moreover, using a histochemical approach, it was evident that deltamethrin at 25 mg/kg was able to produce cell death in the thymus of treated animals 72 hr after treatment. In the present work, we found that cell death was apoptotic in nature as noted first by the inhibition of deltamethrin-induced cell death by aurintricarboxylic acid, an inhibitor of apoptosis, and second, by internucleosomal DNA fragmentation, a hallmark of apoptosis, produced by deltamethrin in treated animals as well in thymocyte suspensions. In addition, the involvement of the Ca/CaM-dependent protein phosphorylation-dephosphorylation cascade in the induction of apoptosis by deltamethrin was supported by the protective role of the calmodulin inhibitor trifluoperazine against the apoptotic effect of deltamethrin on thymocyte suspension. Our results suggest that deltamethrin induced thymus atrophy and altered the Ca/CaM-dependent protein kinase-phosphatase cascade, which might induce programmed cell death. BIOCHEM PHARMACOL 51;4:447–454, 1996.

KEY WORDS. deltamethrin; thymus atrophy; apoptosis; protein dephosphorylation; mice

Synthetic pyrethroids are powerful neuroactive compounds [1]. They have been shown to increase the release of neurotransmitters [2], affect protein phosphorylation [3, 4], and increase the release of intrasynaptosomal Ca^{2+}, thereby elevating the concentration of intrasynaptosomal free Ca^{2+}. A recent report has also shown that they stimulate the production of IP_3[‖] and the translocation of protein kinase C from cytosolic sites into the plasma membrane of rat brain synaptosomes [5]. It was reported originally from our laboratory that deltamethrin, a type II synthetic pyrethroid insecticide, specifically inhibits calcium-calmodulin-dependent protein phosphatase (CaN) in rat brain synaptosomes [6].

To our knowledge, no biochemical studies have been carried out to address the immunotoxicological effects of synthetic pyrethroids. The cells of the immune system provide numerous examples of programmed cell death [7]. Apoptosis, or programmed cell death, is a physiological process of cell death characterized by endogenous endonuclease activation that is involved in normal cell turnover, hormone-induced tissue atrophy, effector cell-mediated target cytolysis, and tumor regression [8]. Apoptosis in thymocytes is strictly dependent upon ongoing protein and mRNA synthesis. Thus, cycloheximide and actinomycin D, blockers of translation and transcription activity, respectively, inhibit both DNA fragmentation and cell death in thymocytes exposed to glucocorticoid hormones [9], Ca^{2+} ionophores [10], and the environment contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [11]. The triphenylmethane dye AuTC is a general inhibitor of nucleases *in vitro* [12]. It has been used successfully to prevent

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‖ *Abbreviations*: IP_3, inositol 1,4,5-triphosphate; IP_2, inositol 1,4-diphosphate; $[Ca^{2+}]_i$, intracellular free calcium, CaN, calcineurin; CaM-kinase, calcium-calmodulin-dependent protein kinase; CaM, calmodulin; TFP, trifluoperazine; AuTC, aurintricarboxylic acid; Con A, concanavalin A; IL-2, interleukin-2; and PCD, programmed cell death.

Received 20 February 1995; accepted 28 August 1995.

degradation during nucleic acid isolation [13], and we have found it to be quite effective in inhibiting endogenous endonuclease activity in neurogenic PC12 cells.¶ The mechanism involved in cell injury and death has been the object of intense investigation in recent years.

It has been reported recently that NFAT is a transcriptional factor that regulates expression of the cytokine IL-2 [14]. The DNA-binding specificity of NFAT is conferred by NFATp, a phosphoprotein that is a target for the immunosuppressive compounds cyclosporin A (CsA) and FK506. It was found that, NFATp is a substrate for CaN *in vitro* [15, 16] and is thought to be dephosphorylated by CaN in activated T-cells, resulting in its translocation from the cytoplasm to the nucleus [17]. The inhibition of CaN by immunosuppressive agents (CsA and FK506) [18] blocks the dephosphorylation of NFATp [15] and the appearance of NFAT in nuclear extracts of stimulated T-cells, which might account for the immunosuppression caused by CsA and FK506 [17].

The abundant expression of CaM-dependent protein kinase in thymocytes points to a potentially important function of this enzyme in lymphocyte Ca^{2+} signaling. This enzyme has a wide tissue distribution and is present in all eukaryotic systems [19, 20]. Several characteristics of CaM-kinase are compatible with its role in mediating hormone action in a variety of cells [19] and in the action of pyrethroids in neuronal cells [4].

Since Ca/CaM-dependent protein phosphatase (CaN)/kinase are indispensible enzymes in the differentiation process of T-cells, we decided to examine the *in vivo* and the *in vitro* effects of deltamethrin on the thymus from the perspective of its effects on the Ca/CaM-dependent protein phosphorylation-dephosphorylation cascade and the role of the Ca^{2+} signaling pathway in thymus atrophy and DNA fragmentation that might lead to programmed cell death. Our results show that deltamethrin induced cell death associated with DNA fragmentation (probably by endogenous endonuclease) in the thymus glands of *in vivo* treated animals as well as in thymocyte suspensions. We discuss how Ca/CaM-dependent protein phosphatase in this process suggests a mechanism for the regulatory role of deltamethrin on cell sensitivity to apoptosis.

MATERIALS AND METHODS

Chemicals

[^{32}P]ATP (5000 Ci/mmol) and *myo*-[2-^{3}H]inositol (1 mCi/mL; 18.2 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). Deltamethrin-1*R* [(2)-α-cyano-3-phenoxybenzylcis-(1*R*,3*R*)-2,2-dimethyl-3-(2,2-dibromovinyl) cyclopropane carboxylate] and deltamethrin-1*S* were gifts from Roussel Uclaf. All other biochemicals were purchased from the Sigma Chemical Co. (St. Louis, MO) and were of the highest purities available.

Animals

Six- to eight-week-old (20–25 g) male mice (Balb/c) were used throughout the study. Animals were housed in suspended stainless steel cages and provided with food and water *ad lib*. All animals were maintained on a 12-hr light/12-hr dark cycle at constant temperature (72 ± 1°F) and humidity (approx. 70%). *In vivo* treatments were made by a single i.p. injection of deltamethrin in corn oil:ethanol (4:1); control animals received an equal volume of vehicle only. Five animals were used for each treatment point.

Effects of Deltamethrin (1R) on Thymus and Body Weight

To study dose–response relationships, five animals were treated with a single i.p. injection of one of the test deltamethrin doses (6, 12, 25 and 50 mg/kg); the thymus was removed after 24 hr, and its weight, as well as the whole body weight, was measured. The time–course effect of deltamethrin was studied by measuring animals held for 1, 2, 3, 5, 14, 28, and 35 days (five animals per each time point) after a single 25 mg deltamethrin/kg dosing (i.p.).

Effects on Phosphoinositide Metabolism

The thymus gland from each treated (25 mg deltamethrin/kg) or untreated mouse was removed and used for *in vitro* incubation experiments to measure inositol metabolism. Slices from each thymus (25–40 mg) were washed for 30 min with Krebs–Ringer buffer at 37°. The slices were removed and incubated with 5 μCi of *myo*-[2-^{3}H]inositol for 90 min at 37°. Prelabeled tissues were washed thoroughly for 60 min at 37° with Krebs–Ringer buffer, pH 7.5, containing a 10 mM concentration of unlabeled *myo*-inositol. The reactions were terminated by the addition of 1 mL of chloroform:methanol (1:2) [5, 21]. The labeled metabolic products were extracted and eluted from columns, each containing 1 mL of Dowex-1 (X8, formate form, 200–400 mesh) as described by Downes and Michell [22], Berridge *et al.* [21], and Enan and Matsumura [5].

Preparation of Cell Suspensions

Thymocyte suspensions were prepared from thymus glands from untreated male mice and maintained exactly as described by McConkey *et al.* [23]. The glands were minced in ice-cold RPMI 1640 medium supplemented with 1% (w/v) BSA, pH 7.2. Thymocytes were passed through two layers of gauze and diluted to a final concentration of 50×10^6 cells/mL of RPMI 1640 [23] before incubating at 37° in a humidified incubator under an atmosphere of 5% CO_2.

Assessment of Cell Viability

Thymocyte samples from five different animals were prepared and used separately for each treatment. The samples were incubated for 24 hr in the presence and absence (control) of deltamethrin (50 μM), Con A (10 μg/mL), the endonuclease inhibitor AuTC (100 μM) [12, 13], and the calmodulin inhibitor TFP (100 μM) [3]. Cell death was measured by trypan blue exclusion. The data are expressed as the means ± SD of five independent experiments.

¶ Enan E and Matsumura F, Manuscript submitted for publication.

Measurement of DNA Fragmentation

Thymus glands from deltamethrin-treated (25 mg/kg, 24 hr) or control animals were removed and examined for evidence of internucleosomal DNA cleavage, using the method of Wyllie [9]. Briefly, thymus glands were homogenized in lysis buffer containing 5 mM Tris-HCl, 0.5% Triton X-100, pH 8.0, and 20 mM EDTA to prevent further nuclease activity. Samples were then centrifuged for 20 min at 13,000 *g* to separate high-molecular-weight chromatin (pellet) from cleavage products (supernatant) [24]. The supernatant containing low-molecular-weight soluble DNA (fragmented DNA) and the pelleted DNA were digested with RNase (100 μg/mL) at 37° for 1 hr. The samples were then deproteinized with proteinase K (200 μg/mL) at 50° for 2 hr followed by phenol extraction and concentrated by ethanol precipitation. DNA contents in pellet and supernatant fractions were determined using the diphenylamine reagent [25]. DNA fragmentation was expressed as a percentage of total DNA in each sample that resisted sedimentation at 13,000 *g* [24, 26, 27]. In parallel experiments, thymocyte suspensions (50×10^6 cells/mL) were incubated for 24 hr in RPMI 1640 medium in the presence of 10 μg/mL Con A or 100 μM AuTC or TFP (100 μM) with and without deltamethrin (50 μM) followed by quantitation of DNA fragmentation.

Measurement of Cytosolic Ca^{2+} Concentration

Thymocytes were preincubated in Krebs buffer with 0.5% BSA for 15 min at 25° before loading with 5 μM Fura-2/AM for 45 min. The cells were then pelleted by centrifugation and resuspended in 1 mL of Krebs buffer. Cell suspensions were incubated with either solvent (less than 1% ethanol) or deltamethrin (50 μM) for 10 min at 37° before the assay, which was performed using a computerized spectrofluorometer (PTI). The R_{max} and R_{min} were determined following treatment with 3 μM 4-Br A23187 (maximum) and 20 mM EGTA (minimum). All measurements were made at excitation wave lengths of 340 and 380 nm and at an emission wavelength of 510 nm. K_d was taken to be 135 nM from the standard curve for Ca^{2+}. The free $[Ca^{2+}]_i$ was measured and computed exactly as described by the supplier (PTI). In some experiments, the effects of Con A (10 μg/mL) and AuTC (100 μM) on $[Ca^{2+}]_i$ mobilization were tested in the presence of deltamethrin. All data are expressed as the means ± SD of five independent experiments.

Measurement of Ca/CaM-Dependent Protein Phosphorylation–Dephosphorylation in In Vivo *Treated Mice*

Male mice (Balb/c) were treated i.p. with 25 mg deltamethrin/kg body weight. Control animals received the same volume of vehicle (corn oil:ethanol, 4:1). After 24 hr, animals were killed, and the thymus glands were removed and homogenized in 50 mM HEPES, pH 7.4, with 1 μg/mL of the protease inhibitors leupeptin, aprotinin, chemostatin and 1 mM phenylmethylsulfonyl fluoride. The nucleus and insoluble materials were pelleted by centrifugation at 2000 *g* for 10 min at 4°. Aliquots of the supernatant (100 μg protein) from control and treated samples were incubated with 0.5 mM EGTA, 0.8 mM $CaCl_2$, 25 nM CaM and HEPES buffer (50 mM, pH 7.4) in a final volume of 80 μL. After 3 min at 37°, the reaction was initiated by the addition of 1 μCi [γ-^{32}P]ATP (1 μM final concentration), and the reaction was stopped after 15, 60, 600 and 1800 sec by the addition of 40 μL of 4X-SDS treatment buffer [3]. The samples were then heated at 95° for 3 min, and each entire volume of the reaction mixture was transferred to a well of 10% SDS–PAGE. The gel was electrophoresed and analyzed using a computerized radioscanner (SyncMaster 3). The data are expressed as the means ± SD of three independent experiments.

Immunohistochemistry for Apoptotic Labeling of Thymic Cells

The thymus glands from four mice in each deltamethrin-treated and control group were removed and fixed by immersion in 1% paraformaldehyde for 3 hr and embedded in paraffin. Sections 5–6 μm thick were cut on a rotary microtome, were deparaffinized in three changes of xylene for 5 min each, and were hydrated in decreasing concentrations of ethanol. Sections were treated with 3% hydrogen peroxide in methanol to eliminate endogenous peroxidase and subsequently washed in PBS. DNA fragmentation in cells was tagged with digoxigenin-dUTP (ApopTag Kit, Oncor). Sections were washed three times in PBS before incubation for 30 min with the secondary antibody anti-digoxigenin-peroxidase. Excess antibody was removed by washing three times in PBS. The peroxidase substrate SG (Vector) was used to complex with cell-bound peroxidase to form a purple-gray reaction product that could be visualized microscopically. Excess SG was removed from the sections by washing in distilled water, and sections were counterstained with nuclear fast red. Control slides were prepared by eliminating the catalytic step needed for tagging positive cells with digoxigenin-dUTP.

Morphometric Analysis of Tissue Sections

The thymus from four animals in each control and treatment group was examined. Ten fields from the cortex and ten fields from the medulla of each animal were randomly captured as digital images using the 40× objective on an Olympus BH-2 microscope interfaced to a MacIntosh IIci computer via a Dage MTI video camera. The degree of cellularity was determined for each field along with the proportion of apoptotic positive cells. Since the cellularity of the cortex and medulla, respectively, was found to be similar in treated and control animals, the percentage of apoptotic-positive cells was determined as the areal fraction of the total number of fields analyzed. To quantify the frequency of apoptotic cells, the software program NIH Image was used to highlight the labeled cells captured, employing a density gradient scale. The density gradient was calibrated to highlight only positive cells to permit the distinction and quantitation of positive cells compared with non-

positive cells and stroma throughout the thymus. Areas highlighted were counted by pixel number and compared with the total number of pixels within each field. The total number of apoptotic cells per cm^2 of cortex was also determined, viewing five fields per animal.

RESULTS

Dose-Response and Time-Course Effects of Deltamethrin on Thymus and Body Weight

The 24-hr dose effect of *in vivo*-administered deltamethrin on thymus weight is shown in Fig. 1A. A significant thymic involution (atrophy) effect was noted at a dose as low as 6 mg/kg. It was apparent that the effect of deltamethrin was dose dependent, with a reduction in thymus weight of 22, 32, and 41% following single injections of 6, 25, and 50 mg/kg of deltamethrin, respectively. Based on this experiment, 25 mg/kg was chosen as a standard dose for all subsequent studies. Time-course effects of deltamethrin on thymus and body weight also were studied. As shown in Fig. 1B, thymus atrophy developed rapidly, beginning on day 1, with the maximal effect occurring 2 weeks after deltamethrin treatment (25 mg/kg). No significant effect on body weight was noted during the course of the study (Fig. 1C). This time point (24-hr treatment) was utilized for the *in vivo* experiments that follow. As illustrated in Table 1, deltamethrin-1*R* significantly reduced thymic weight 24 hr after a single i.p. administration of 25 mg/kg. Deltamethrin-1S produced only modest effects.

Effect of Deltamethrin on Cell Viability, DNA Fragmentation and $[Ca^{2+}]_i$ Mobilization

Within 24 hr of treatment, deltamethrin induced cell death in thymocyte suspensions to approximately 45%. To assess the involvement of internucleosomal DNA cleavage in this process, we first measured the DNA fragmentation in thymus glands of deltamethrin-treated animals. Second, the endonuclease inhibitor, AuTC, was added to the thymocyte suspensions 10 min prior to the addition of deltamethrin. It was evident that deltamethrin induced 55% DNA cleavage in the thymus glands after 24 hr treatment (Table 2), which is a characteristic of apoptosis. AuTC, however, antagonized both the cell killing and DNA cleavage, which were induced by deltamethrin within the 24-hr treatment. On the other hand, the calmodulin inhibitor TFP partially antagonized the cytotoxicity of deltamethrin on thymocyte suspensions (Table 2). Deltamethrin also induced $[Ca^{2+}]_i$ mobilization in thymocyte suspensions, and AuTC antagonized this effect (Table 2). To our surprise, Con A significantly induced $[Ca^{2+}]_i$ mobilization but did not induce cell death or DNA fragmentation in thymocyte suspensions (Table 2).

In Vivo *Effect on Inositol Pathway*

The active form of type II synthetic pyrethroids, deltamethrin-1*R*, produced a significant increase ($P \leq 0.01$) in intracellular

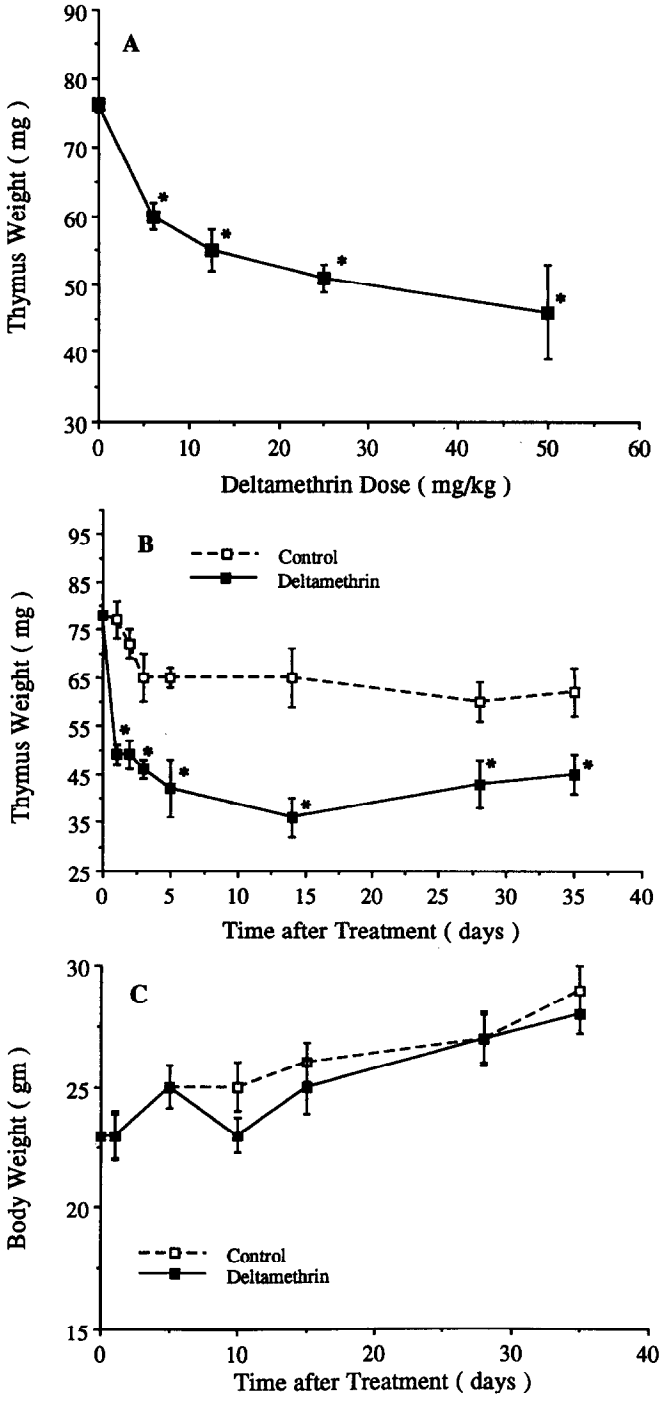

FIG. 1. Dose- and time-response of deltamethrin on thymus involution of male Balb/c mice. (A) Different doses of deltamethrin were injected i.p. After 24 hr, the thymus was removed and weighed. (B) A single dose of deltamethrin (25 mg/kg) was injected i.p. for different time intervals. Treated and untreated (control) mice were killed at each time point, and the thymus was removed and weighed. (C) Body weight for treated and untreated mice with 25 mg/kg of deltamethrin. Control animals received an equivalent volume of vehicle only. Data are expressed as means ± SD of 5 animals. An asterisk denotes a statistically significant difference compared with control ($P \leq 0.05$).

TABLE 1. *In vivo* effect of 25 mg/kg of some insecticides on mouse thymus and body weight after 24 hr of i.p. treatment

	Thymus weight (mg)	Body weight (g)	Thymus/body wt ($\times 10^{-3}$)
Control	76 ± 1.1	33 ± 0.8	2.303
Deltamethrin-1R	40 ± 1.9*	32 ± 1.0	1.250*
Deltamethrin-1S	62 ± 2.8	33 ± 0.7	1.875

Data are the means ± SD of 3 experiments; five animals were used in each expriment.
* Significantly different from the control $P \leq 0.01$ (Cochran *t*-test).

phosphoinositide levels in thymus, particularly IP_3 and IP_2, 24 hr after a single treatment (25 mg/kg) (Table 3).

Effect of Deltamethrin on Ca/CaM-Dependent Protein Dephosphorylation

Thymic extract samples (100 μg protein) from deltamethrin-treated and untreated mice were phosphorylated using [γ-^{32}P]ATP in the presence of Ca^{2+} and CaM. The phosphorylation was stopped by the addition of 4X-SDS treatment buffer at different times, and the samples were analyzed using SDS–PAGE. The data in Fig. 2 show that: (1) deltamethrin induced protein phosphorylation as early as a 15-sec incubation with Ca/CaM and [γ-^{32}P]ATP, and (2) the rate of dephosphorylation was slower in deltamethrin-treated samples than in the control over a 30-min incubation period with Ca/CaM. It was found that after a 15-sec incubation with Ca/CaM and [γ-^{32}P]ATP a 7% increase in protein phosphorylation was observed in deltamethrin-treated samples over the control samples. This difference was increased markedly over the time of the incubation with Ca/CaM. After 10 min, for instance, a 55% increase in the phosphoproteins was observed in deltamethrin-treated samples over the control. Such an increase in the percentage of protein phosphorylation in treated samples is due to the rapid decline in the phosphoproteins in control samples rather than to an increase in protein phosphorylation in deltamethrin-treated samples. This rapid decline in phosphorylated proteins in the control samples was paralleled to a steady-state level in deltamethrin-treated samples over the incubation time with Ca/CaM, i.e. the rate of protein dephosphorylation was substantially faster in control compared with treated samples. For example, after 15 sec, 12,700 ± 250 dpm ^{32}P incorporated into the proteins of control samples. After 10 min, a 33% decline in this value was observed (8,500 ± 410 dpm). In deltamethrin-treated samples 13,200 ± 610 dpm was measured as phosphoproteins after a 15-sec incubation with Ca/CaM and [γ-^{32}P]ATP. An approximately 1% decline was observed after a 10-min incubation followed by an 11% decline after a 30-min incubation with Ca/CaM and [γ-^{32}P]ATP (Fig. 2). From these data it was obvious that deltamethrin inhibited protein dephosphorylation (Ca/CaM-dependent protein phosphatase) in thymus glands of treated animals.

Immunohistochemistry

Distinction of the thymic cortex from the medulla was based on location and relative cellularity of the tissue. The cortex contained significantly more cells than the medulla. Although the cellularity of each region was significantly different, the number of apoptotic-positive cells per unit area in the cortex and medulla of treated animals was on the average higher than that seen in controls. The percentage of area from ten cortical fields per animal containing apoptotic-positive cells was 4.4% in treated animals and 2.3% in control animals (Fig. 3). In the medulla, the percentage of area containing labeled cells was 2.1 and 1.6% in treated and control animals, respectively. However, due to the large variations in treated animals, the differences between control and treated animals were not statistically significant. The average number of apoptotic-positive cells per cm^2 was 425 (ranged from 150 to 800) in treated animals and 311 (ranged from 190 to 315) in control animals. This difference represented an average of 37% greater numbers of apoptotic-positive cells in treated animals than in control animals. The frequency of positive cells was higher in the cortex than in the medulla; however, this was due in large measure to the greater cellularity of the cortex.

DISCUSSION

Our current work has been concerned with identifying the possible biochemical mechanisms involved in thymocyte PCD. Glucocorticoid hormones [9] and Ca^{2+} ionophore [10] stimulate the process, resulting in extensive DNA fragmentation and cell death. Many others have shown that thymocyte DNA fragmentation and cell death are dependent on an early sustained increase in cytosolic Ca^{2+} concentration [24, 26].

TABLE 2. Effect of deltamethrin on $[Ca^{2+}]_i$, cell viability, and DNA fragmentation

	$[Ca^{2+}]_i$* (nM)	DNA fragmentation†‡ (%)	Cell death§ (%)
Control	77 ± 5	7 ± 0.9†	5 ± 0.7
Deltamethrin-1R	100 ± 8‖	55 ± 3.0†‖	45 ± 4.0‖
Con A	225 ± 9‖	5 ± 0.6‡	2 ± 0.1
Con A plus deltamethrin-1R	240 ± 15‖	52 ± 4.0‡‖	47 ± 3.0‖
AuTC plus deltamethrin-1R	80 ± 10	23 ± 2.0‡‖	15 ± 0.9‖
TFP plus deltamethrin-1R	ND¶	31 ± 2.0‡‖	26 ± 1.5‖

Data are the means ± SD of 5 independent experiments.
* All $[Ca^{2+}]_i$ assays were measured in thymocyte suspensions after 10 min of simultaneous treatment with deltamethrin in the presence and absence of other test chemicals, as described in Materials and Methods. Control samples received the same volume of vehicle only (less than 1% ethanol).
†‡ DNA fragmentation was measured by two methods: (†) in thymus glands that were removed from control and deltamethrin-treated animals following 24 hr of treatment; or (‡) Con A (10 μg/mL), AuTC (100 μM), or TFP (100 μM) was added to the thymocyte suspensions 10 min prior to deltamethrin. See Materials and Methods for further details. The data of DNA fragmentation obtained in the presence of Con A only were considered as a control for the treatment with other chemicals that follow in this table.
§ All cell viability assays were measured in thymocyte suspensions after 24 hr of treatment, as described in Materials and Methods.
‖ Significantly different from the corresponding control value, $P \leq 0.01$ (Cochran *t*-test).
ND = not determined.

TABLE 3. *In vivo* effect of certain insecticides on the inositol pathway in the mouse thymus

	Radioactivity (dpm)					
	Inositol	Glycerophosphoinositol	InsIP	Ins(1,4)P_2	Ins(1,4,5)P_3	Ins(1,3,4,5)P_4
Control	66,660 ± 1,960	3,000 ± 240	1,366 ± 158	1,758 ± 180	2,444 ± 172	239 ± 14
Deltamethrin-1R	139,700 ± 1,480*	5,448 ± 172*	3,058 ± 134†	3,884 ± 144*	4,032 ± 248*	449 ± 56
Deltamethrin-1S	121,090 ± 5,988*	5,184 ± 336*	2,299 ± 137†	2,663 ± 377	3,180 ± 920	252 ± 30

Values are means ± SD, N = 3 independent experiments. A single treatment (25 mg/kg) was given.
*† Significantly different from control values; *$P \leq 0.01$ and †$P < 0.05$ (Cochran *t*-test).

The magnitude of the increased Ca^{2+} appears to be rate-limiting for endonuclease activation in ionophore-treated cells, and DNA fragmentation is most likely the lesion that directly mediates cell death [27]. Cell proliferation and cell death are both continuous processes normally taking place in the thymus. To our knowledge, the effect of deltamethrin on thymus atrophy in the intact thymus has not been reported. In the present work, we found that deltamethrin induced thymus atrophy and increased $[Ca^{2+}]_i$ in treated mice (Fig. 1 and Table 2). This phenomenon may be attributable to: (a) inhibition of cell proliferation, (b) excessive cell death, and/or (c) an increase in the export of the thymocytes to peripheral tissues. Our study was concerned with the possibility that deltamethrin induced thymus atrophy via apoptosis. From our data it was evident that deltamethrin induced 45 ± 4% cell death in thymocyte suspensions, and this was associated with 55 ± 3% breakage of DNA after 24 hr of treatment, which is a characteristic of apoptosis. In control samples, the corresponding values were 5 ± 0.7 and 7 ± 0.9%, respectively (Table 2).

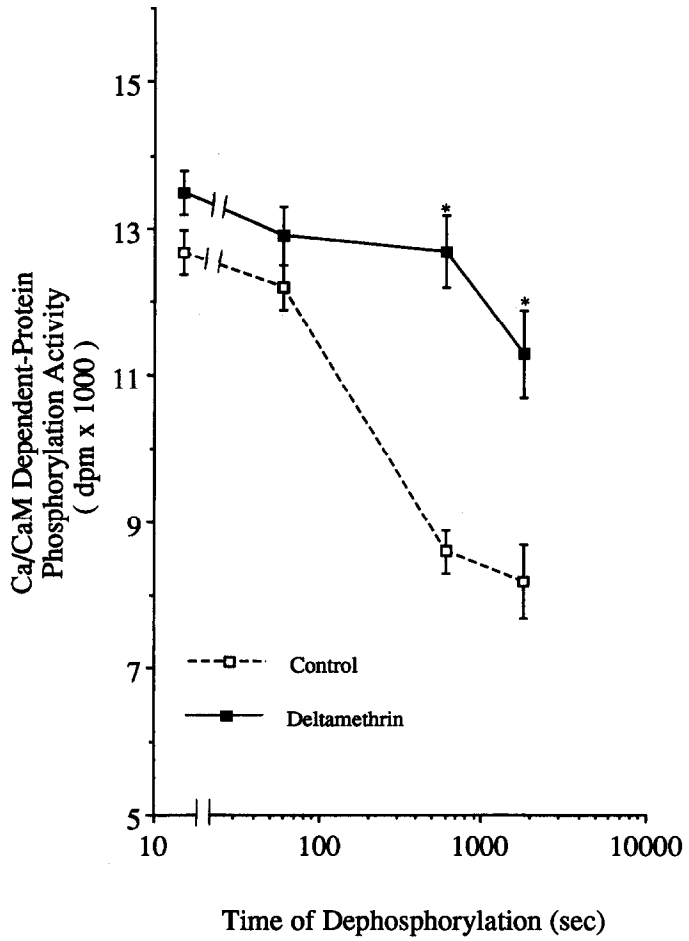

FIG. 2. Deltamethrin effects on protein phosphorylation/dephosphorylation in thymus. Protein (100 μg) aliquots of 10,000 *g* supernatant of thymus from treated and untreated mice were incubated with Ca/CaM as described in Materials and Methods. The reaction was stopped at different times from 15 to 1800 sec, and the phosphoproteins were analyzed using SDS–PAGE. The dried gel was scanned using a computerized radio scanner (SyncMaster 3). Data are the means ± SD of 5 animals for each point. An asterisk denotes a statistically significant difference compared with control ($P \leq 0.05$).

One critical question that arose during the current work was whether such an increase in the Ca^{2+} level in thymocytes would result in PCD. It is well known that T cell proliferation is also mediated by a sustained Ca^{2+} increase [28], implying that all of the PCD-sensitive cells would be committed to die within the thymus. Independent reports have supported a critical role for $[Ca^{2+}]_i$ in thymocyte apoptosis. The apoptotic effects of glucocorticoids involve Ca^{2+} influx [29] and can be caused by A23187 (calcium ionophore). In addition, a nuclear Ca^{2+}-dependent and Zn^{2+}-sensitive endonuclease, whose activity appears to be responsible for DNA fragmentation, has been observed in glucocorticoid-treated thymocytes [30]. Wyllie *et al.* [10] reported that A23187 induces both DNA fragmentation and morphological alterations typical of glucocorticoid-induced thymocyte apoptosis. In the current work, we have shown that deltamethrin induced both DNA fragmentation and an increase in $[Ca^{2+}]_i$. Because DNA fragmentation may be directly involved in triggering cell death in apoptotic thymocytes [24, 26, 27, 31], our current findings support the idea that such an increase in $[Ca^{2+}]_i$ may play a role in cell death through apoptosis as a result of deltamethrin treatment. However, we found that the lectin Con A induced a sustained and significant $[Ca^{2+}]_i$ mobilization in thymocytes and that did not result in DNA fragmentation (Table 2). Thus, caution must be exercised in interpreting the above data on $[Ca^{2+}]_i$ mobilization only.

Another important question that has been addressed during the current work was whether deltamethrin induced Ca/CaM-kinase activity or inhibited the Ca/CaM-dependent protein-dephosphorylation cascade (i.e. CaN). Our interpretation is that deltamethrin likely induced Ca/CaM-kinase (Fig. 2), which might antagonize the action of CaN [32] by phospho-

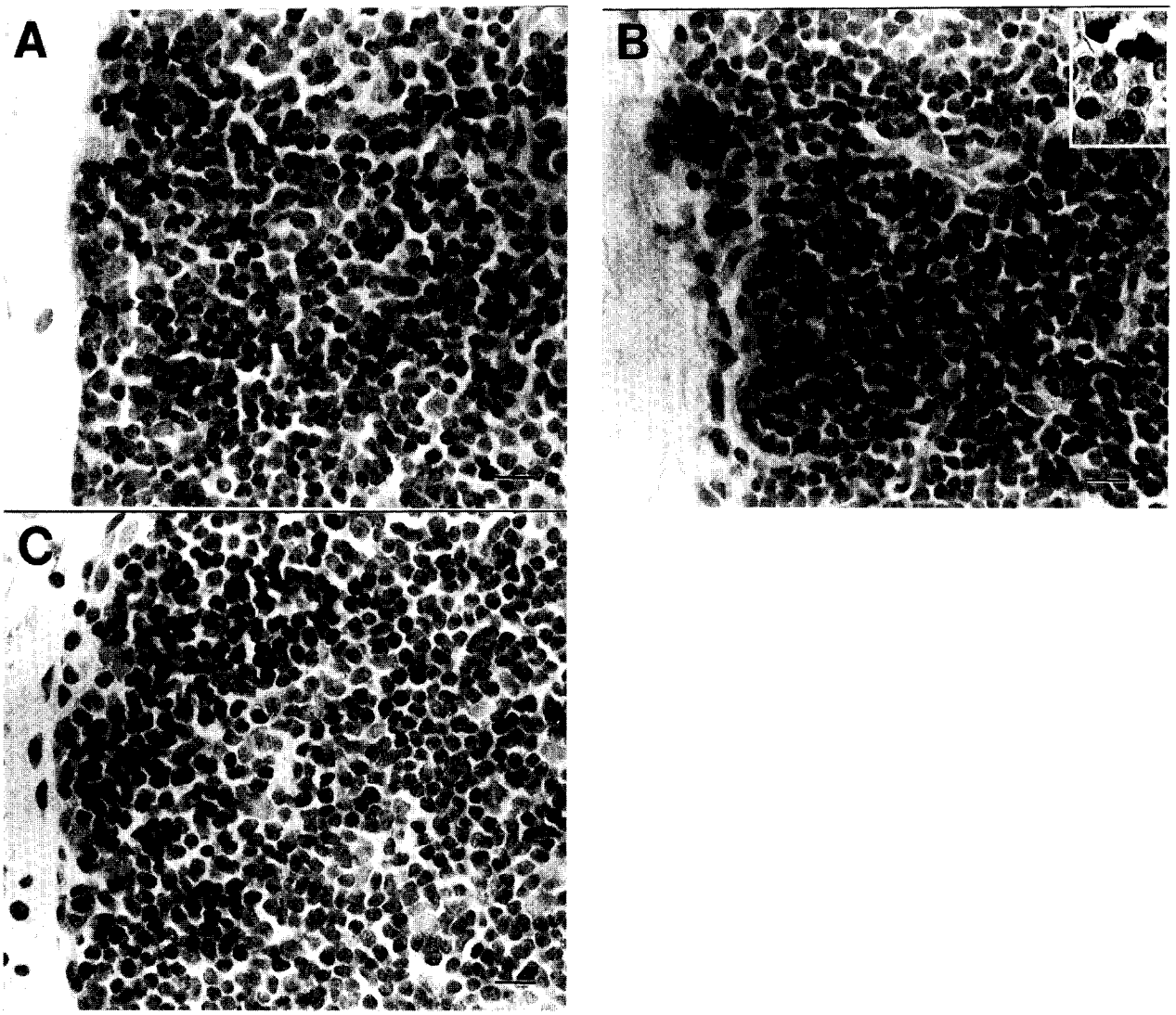

FIG. 3. Light micrographs of the thymic cortex in a control (A) and treated (B) mouse. Apoptotic-positive cells are noted as darkly stained cells (arrows). Negative cells are counterstained with nuclear fast red. The inset in panel B is a higher magnification of a cluster of positively stained cells within the field identified by the arrow. Panel C is the negative control with the catalytic step required for tagging apoptotic cells, with digoxigenin-dUTP eliminated. Scale bar is 10 μm.

rylating critical transcription factors such as NFATp, a cytoplasmic component of nuclear-NFAT that serves as a substrate for CaN [14] and contains 12 consensus phosphorylation sites for Ca/CaM-kinase [15]. In the current work, it was clear that deltamethrin induced Ca/CaM-dependent protein phosphorylation as early as 15 sec after the addition of Ca/CaM and [γ-^{32}P]ATP (Fig. 2). After 30-min, there was a definite increase in protein dephosphorylation in untreated samples and a slight decrease in the level of the phosphoproteins in deltamethrin-treated samples. It is possible to assume that deltamethrin induced Ca/CaM-kinase in *in vivo* treated animals, which resulted in antagonizing CaN action under *in vitro* phosphorylation conditions. Supporting these findings is the work of Nghlem *et al.* [32] who found that CaM-kinase blocks the ability of constitutive CaN plus phorbol myristate acetate to activate IL-2. They concluded that it was unlikely that this antagonism involved a direct interaction between kinase and phosphatase, as neither phosphorylation of CaN in the CaM-binding site nor possible dephosphorylation of CaM-kinase by CaN would occur with constitutive constructs. However, this antagonism phenomenon between CaM-kinase and CaN in thymus requires further study. Another possibility is that the initial autophosphorylation with Ca/CaM, which we observed after 15 sec in treated and control samples, enables the kinase not only to phosphorylate substrates in the absence of Ca/CaM but to continue autophosphorylating itself. This phase of autophosphorylation decreases CaM binding to the kinase [33] and caps the activity at the level of its Ca^{2+}-dependent activity [34]. This possibility in combination with active CaN might explain the rapid decline in phosphoproteins of control samples. However, such assumptions need further investigation. In addition, the level of CaN and CaM-kinase proteins should be addressed. Other transcription factors such as AP-1, NF-κB and NFAT [35, 36] that contribute significantly to the activity

of the mouse IL-2 promoter should be considered in future studies.

This work was supported by ES05233, ES03575, and ES05707 from the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

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