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Pyrethroid Insecticides as Phosphatase Inhibitors

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ABSTRACT. In this study we tested the hypothesis that pyrethroid insecticides inhibit calcineurin directly and that inhibition is unaffected by the immunophilin cofactors necessary for calcineurin inhibition by cyclosporin A and FK506. The type II pyrethroid insecticides *cis*-cypermethrin (c-Cyp), *trans*-cypermethrin, deltamethrin (Delt), and fenvalerate Aα (Fen), as well as the type I pyrethroid insecticides *cis-* and *trans*-permethrin and *S*-bioallethrin, were unable to inhibit the phosphatase activity of purified calcineurin under conditions of maximal activation by Ca^{2+} and calmodulin. Furthermore, c-Cyp, Delt, and Fen did not affect the Ca^{2+} dependence of calcineurin at 0.1 μM of calmodulin, indicating that Ca^{2+} binding to calmodulin was not affected by these agents. c-Cyp, Delt, and Fen also failed to inhibit calcineurin phosphatase activity in rat brain supernatant and cultured IMR-32 cells, although potent inhibition was displayed by both cyclosporin A and FK506 in each of these systems. Neither the Ca^{2+}-dependent nor the okadaic acid-inhibitable phosphatase activity toward a 24-amino acid ^{32}P-phospho-peptide substrate was affected by any of the pyrethroid insecticides, indicating that neither type-1 or type-2A phosphatase nor calcineurin is inhibited by pyrethroids. To determine if these results were dependent upon experimental conditions, experiments were repeated using polyethylene glycol-treated glass tubes in place of the standard polypropylene tubes. Regardless of the type of tube, no inhibition of calcineurin by any of the pyrethroid insecticides was observed. These data indicate that the pyrethroid insecticides are not effective inhibitors of calcineurin or other phosphatases. BIOCHEM PHARMACOL **55**;12:2017–2022, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. calcineurin; type II pyrethroids; insecticides; phosphatase; calcium; calmodulin

The pyrethroid insecticides are widely used and, in combination with the organophosphates, have largely supplanted the chlorinated hydrocarbons (e.g. DDT[‖]) as insecticides. Although the pyrethroids are much less toxic to mammals than are many other pesticides, they are not free from adverse effects [1]. The primary target of the pyrethroids is the sodium channel [1–4], and these compounds exert a profound effect on sodium channel gating at low micromolar concentrations [5]. Other channels have been reported to be affected by the pyrethroids; however, the importance of pyrethroid actions at sites other than the Na^+ channel is questionable as the pyrethroids are only effective at high micromolar concentrations on other channels [3, 6]. In addition to the direct effects of the pyrethroids on ion channels, the type II pyrethroid insecticides stimulate neurotransmitter release, which cannot be abolished with the sodium channel blockade produced by tetrodotoxin [7, 8], indicating that an additional mechanism may be involved in type II pyrethroid actions. The ability of the pyrethroids to exert effects beyond those documented on ion channels has caused other mechanisms of action to be proposed, including the modulation of calmodulin actions [9], inhibition of the phosphatase calcineurin [10], and activation of a calmodulin-dependent kinase [11] and protein kinase C pathways [12]. The actions of pyrethroids on calcineurin are particularly significant for understanding the potential for immunotoxicity of these compounds because calcineurin activity is an absolute requirement for the generation of an immune response [13, 14]. The finding that type II pyrethroid insecticides are extremely potent inhibitors of calcineurin [10] suggests a possible mechanism for immunotoxicity of these compounds. Although controversial, the immunotoxicity of pesticides, including the type II pyrethroid insecticide cypermethrin [15], has been reported [11, 15, 16]. Therefore, determining the mechanism by which these insecticides inhibit calcineurin is important from the perspective of studying immunosuppression.

Calcineurin is unique in being the only Ca^{2+}- and calmodulin-dependent protein phosphatase [17–19]. Inhibition of calcineurin phosphatase activity by the type II pyrethroid insecticides was demonstrated initially in crude tissue extracts [20]. Specifically, deltamethrin treatment of isolated synaptic membranes resulted in increased levels of protein phosphorylation as compared with untreated con-

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‖ *Abbreviations:* DDT, dichlorodiphenyltrichloroethane; and cAMP, cyclic AMP.

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trol samples. Although these results could be explained by either an inhibitor of calcineurin or a more general inhibitor of calmodulin, it is unlikely that calmodulin is the target, considering that the insecticides have been shown to be effective inhibitors of protein dephosphorylation at submicromolar concentrations [20]. These studies also did not determine if the pyrethroids are specific for inhibiting calcineurin or if they would be effective inhibitors for other phosphatases. Further experiments showed that purified calcineurin is inhibited by the pyrethroids [10], with the activity of calcineurin toward the small chemical substrates *para*-nitrophenylphosphate and phosphotyrosine being inhibited by several of the type II pyrethroid insecticides at subnanomolar concentrations. Mechanistically, this ability is in sharp contrast with the clinically important calcineurin inhibitors cyclosporin A and FK506, neither of which can inhibit isolated calcineurin as both compounds are dependent upon protein cofactors and Ca^{2+} in order to bind to the phosphatase. Thus, the unique ability of the pyrethroids to directly inhibit calcineurin has led to these compounds being viewed both as a tool for dissecting out the details of calcineurin activity and as a potential mechanistic explanation for some of the toxic effects of pyrethroids. As tools, the pyrethroids are now being utilized as calcineurin inhibitors by several investigators [21–30] without direct measurement of their efficacy as calcineurin inhibitors. This acceptance of pyrethroid actions on phosphatases, however, is not universal as this ability has been questioned recently [1].

In the present study, the ability of seven pyrethroid insecticides to inhibit calcineurin activity toward a model peptide substrate was assessed using three different systems: a reconstituted system using purified calcineurin and calmodulin; a tissue homogenate system using supernatant from a rat brain homogenate; and an *in vitro* system using cultured IMR-32 cells. Although the established calcineurin inhibitors cyclosporin A and FK506 both displayed potent inhibition of calcineurin, no effect on calcineurin activity was detected by any of the pyrethroid insecticides tested.

MATERIALS AND METHODS

Materials

Bovine brain calcineurin and bovine testes calmodulin were isolated from frozen tissues as previously described [31, 32]. Rat brains were purchased from Pel-freez, and IMR-32 cells were obtained from the American Type Culture Collection. The synthetic peptide substrate DLDVPIPGRFDRRVSVAAE [33] was synthesized and purified by the University of Nebraska Medical Center Core Protein Laboratory and used as a phosphatase substrate following phosphorylation with ^{32}P by the catalytic subunit of cAMP-dependent protein kinase. Cyclosporin A was provided by Sandoz and FK506 by Fujisawa Pharmaceuticals. The pyrethroid insecticides *cis*-permethrin, *trans*-permethrin, *cis*-cypermethrin, and *trans*-cypermethrin were provided by the FMC Corp.; 1*R*-*cis*-α*S* deltamethrin was provided by Roussel Uclaf; and fenvalerate and *S*-bioallethrin were purchased from Chem Service. All pyrethroids were supplied at greater than 99% purity and were delivered in a minimum volume of 100% ethanol so that the final concentration of ethanol in the reaction mixture did not exceed 0.2% unless otherwise noted.

Determination of Calcineurin Activity

Calcineurin phosphatase activity was assayed as previously described [34]. Phosphorylated substrate was prepared by phosphorylating the serine residue of the synthetic peptide with the catalytic subunit of cAMP-dependent protein kinase (Boehringer Mannheim) and [^{32}P]ATP; the peptide sequence DLDVPIPGRFDRRVSVAAE corresponds to a segment of the R-II subunit of cAMP-dependent protein kinase. Briefly, calcineurin (10 nM) with two molar equivalents of calmodulin or a predetermined amount of brain supernatant or cell homogenate was preincubated for 5 min (0°) in the presence of various concentrations of inhibitor; for vehicle control reactions, an equal volume of ethanol was added. After warming the reactants to 30°, the phosphorylated peptide substrate was added to a final concentration of 1 μM and the dephosphorylation reaction was allowed to proceed for 30 min. The reaction was stopped with 10% trichloroacetic acid, and free ^{32}P was separated from peptide by chromatography over AG-50W-X8 (Bio-Rad) resin. Free ^{32}P in the flow-through solution was quantitated by liquid scintillation counting and reflects the amount of phosphate released from the [^{32}P]-phosphoserine peptide by phosphatase activity. To distinguish calcineurin activity from that of the other major phosphatases, parallel samples were assayed using 1 μM of okadaic acid to inhibit protein phosphatases type-1 and type-2A or 1 mM of EGTA to chelate Ca^{2+} and inhibit calcineurin. Calcineurin phosphatase activity is defined as the proportion of Ca^{2+}-dependent activity insensitive to okadaic acid and was calculated as the difference in activity between the samples containing okadaic acid and the samples containing okadaic acid plus EGTA. In the experiments using purified calcineurin, this enzyme accounts for nearly 100% of phosphatase activity, whereas approximately 60% of observed phosphatase activity is catalyzed by calcineurin in rat brain and IMR-32 cell supernatants.

Specificity for Inhibition of Calcineurin by the Pyrethroids

Selectivity of the type II pyrethroids as calcineurin inhibitors was evaluated by determining the effect of these chemicals on the activity of the other major phosphatases. Because okadaic acid inhibits both type-1 and type-2A phosphatases, the combined activity of these two phosphatases was calculated as the okadaic acid-inhibitable phosphatase activity observed in the presence of 1 mM of EGTA (i.e. the difference between activity observed in

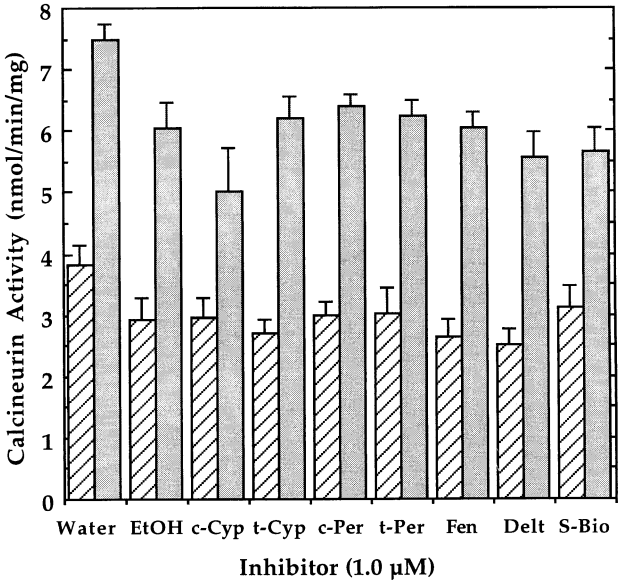

FIG. 1. Calcineurin phosphatase activity in the presence of pyrethroid insecticides. Isolated bovine brain calcineurin was assayed in the presence of 0.1 μM of calmodulin and intermediate (1.0 μM, hatched bars) or maximally stimulating (30 μM, filled bars) Ca^{2+} concentrations. Ethanol (0.33%) as the vehicle control had a modest effect to inhibit the phosphatase at each Ca^{2+} concentration. Seven pyrethroid insecticides: *cis*-cypermethrin (c-Cyp), *trans*-cypermethrin (t-Cyp), *cis*-permethrin (c-Per), *trans*-permethrin (t-Per), fenvalerate Aα (Fen), deltamethrin (Delt) and S-bioallethrin (S-Bio) were tested at 1.0 μM each as phosphatase inhibitors. Differences between groups were assessed by ANOVA, and no significant effects of pyrethroids were determined at either Ca^{2+} concentration. N = 4 or 6; bars represent SEM.

samples containing EGTA and samples containing okadaic acid plus EGTA).

Calcium- and Calmodulin-dependent Inhibition of Calcineurin by Pyrethroids

To determine if inhibition by the pyrethroid pesticides is dependent on Ca^{2+} and calmodulin, various concentrations of these two cofactors were incubated with 10 nM of calcineurin, and the resulting activity toward the ^{32}P-labeled peptide substrate was measured. EGTA (1 mM) was used to buffer free Ca^{2+}, and free Ca^{2+} concentration was calculated using the method of Fabiato and Fabiato [35].

RESULTS

Using a maximally stimulating concentration of Ca^{2+} (30 μM), there was no significant difference in calcineurin activity between vehicle control and pyrethroid-treated samples (Fig. 1, filled bars). To address the possibility that the pyrethroids affect the Ca^{2+} dependence for calmodulin activation of calcineurin, this experiment was repeated using a concentration of calcium equal to its EC_{50} (1.0 μM). As can be seen by the hatched bars (Fig. 1), none of the pyrethroids changed the calcineurin activity supported by 1

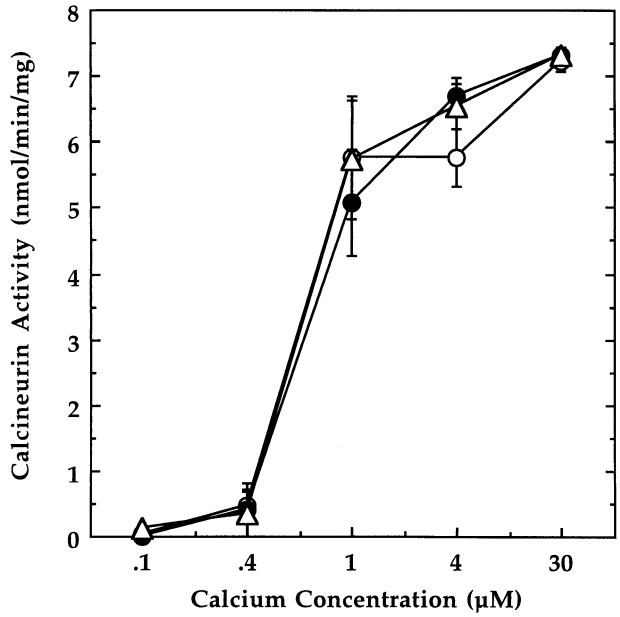

FIG. 2. Ca^{2+} concentration-dependence of calcineurin in the presence of deltamethrin. Purified calcineurin (10 nM) and calmodulin (0.1 μM) were assayed in the presence of 10 nM (●) or 1.0 μM (△) of deltamethrin or vehicle control (○) at the indicated Ca^{2+} concentrations. N = 3; bars represent SEM.

μM of Ca^{2+}, indicating that they did not shift the Ca^{2+} dependence of calcineurin. This result supports the observation that the pyrethroid insecticides have no effect on the phosphatase activity of purified calcineurin.

To verify that the type II pyrethroid insecticides do not act on calmodulin and shift the Ca^{2+} concentration-dependence of calcineurin activation, the full calcium dependence of calcineurin was tested in the presence and absence of deltamethrin, fenvalerate, and *cis*-cypermethrin; these pyrethroids have been reported to be the most potent inhibitors of calcineurin [10]. As shown in Fig. 2, deltamethrin had no effect on the Ca^{2+} dependence of calcineurin nor on the maximal activity of the enzyme at saturating Ca^{2+}. This result was obtained even when the pyrethroids were present in 10-fold excess of calmodulin, indicating that the pyrethroids do not interfere with calmodulin-dependent signaling. Similarly, fenvalerate and *cis*-cypermethrin had no effect on the Ca^{2+} concentration-dependence of calcineurin or on maximum calcineurin activity (data not shown).

Although the above results clearly show that the activity of purified calcineurin is unaffected by the pyrethroid insecticides, it is possible that a protein cofactor (e.g. an immunophilin) is necessary for calcineurin inhibition by the pyrethroids. To address this possibility, the effects of deltamethrin and *cis*-cypermethrin as inhibitors of calcineurin activity were evaluated in the supernatant fraction from rat brain homogenate and in IMR-32 neuroblastoma cells in culture. Cyclosporin A and FK506, each of which requires an immunophilin cofactor for calcineurin inhibition, were used as positive controls. The calcineurin activ-

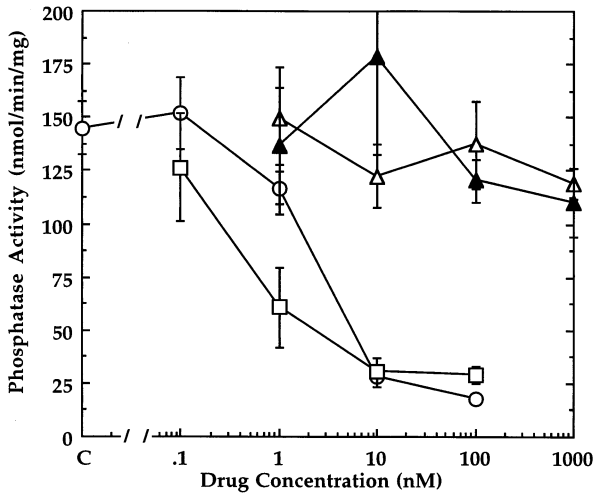

FIG. 3. Inhibition of Ca^{2+}-dependent phosphatase activity in intact IMR-32 cells. *cis*-Cypermethrin (▲), deltamethrin (△), cyclosporin A (○) and FK506 (□) were tested as calcineurin inhibitors in intact IMR-32 neuroblastoma cells. Cells were exposed to the indicated concentration of inhibitor for 2 hr and then were harvested and assayed for calcineurin phosphatase activity. Activity in vehicle-treated control cells is shown at "C". N = 3; bars represent SEM.

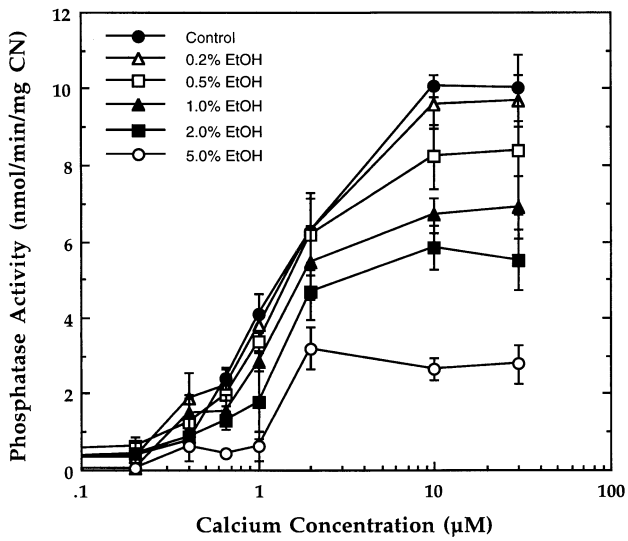

FIG. 4. Inhibition of calcineurin (CN) phosphatase activity by ethanol. Ca^{2+} dependence of calcineurin phosphatase activity in the presence of 0.1 μM of calmodulin was assayed at the indicated concentrations of ethanol. N = 4; bars represent SEM.

ity in cell homogenate prepared from IMR-32 cells treated with deltamethrin or *cis*-cypermethrin (1 nM to 1 μM) showed no difference from that of control cells (Fig. 3). In agreement with this observation, these two pyrethroids had no effect on calcineurin activity when added to brain supernatant (data not shown). In contrast, the established calcineurin inhibitors cyclosporin A and FK506 were each potent inhibitors of calcineurin activity when added to cultured cells (Fig. 3) or to brain supernatant. Taken together, these results indicate that the pyrethroid insecticides 1) do not inhibit purified calcineurin; 2) do not affect calcineurin activity in the presence of cofactors for the established calcineurin inhibitors; and 3) do not inhibit calcineurin activity in intact cells.

To determine if the pyrethroid insecticides can inhibit type-1 or type-2A phosphatases, *cis*-cypermethrin was evaluated for its effect on okadaic acid-sensitive phosphatase activity. *cis*-Cypermethrin at concentrations up to 1 μM had no effect on okadaic acid-sensitive phosphatase activity in IMR-23 cell homogenate (data not shown).

Because it has been reported that polyethylene glycol-coated glass tubes are essential for the calcineurin-inhibitory activity of the pyrethroids, assay conditions also were evaluated. Reactions carried out in coated glass tubes and in plastic tubes exhibited identical calcineurin activity, both in the presence and absence of 0.1 to 1.0 μM of *cis*-cypermethrin, deltamethrin, or fenvalerate (data not shown). These results confirm our finding that phosphatase activity in general and calcineurin activity in particular are unaffected by the pyrethroid insecticides under the assay conditions tested.

Because the data in Fig. 1 showed that calcineurin was inhibited significantly at 0.33% ethanol, the sensitivity of purified calcineurin to this solvent was examined. Figure 4 shows that calcineurin is sensitive to inhibition by ethanol with an IC_{50} of approximately 1%.

DISCUSSION

Calcineurin is a unique phosphatase, both in having a limited substrate specificity and in its regulation by Ca^{2+} and calmodulin. As with any calmodulin-dependent process, actions of inhibitors can be manifest in two ways. Direct inhibitors of the enzyme will inhibit the maximal enzyme activity, whereas inhibitors of calmodulin can inhibit maximal activity or shift the Ca^{2+} concentration-dependence of enzyme activation. This study evaluated the type II pyrethroid insecticides for their ability to inhibit maximal calcineurin activity and to shift the Ca^{2+} concentration–response curve. In experiments examining actions of the pyrethroids on purified calcineurin and calmodulin, the ethanol vehicle at 0.33% final concentration exerted a significant inhibitory effect on the phosphatase activity. However, none of the insecticides tested had any further effect on either the Ca^{2+} dependence of calcineurin or on the maximal activity of the enzyme. These results are in direct opposition to the findings of a previous study reporting potent inhibition of calcineurin by a number of type II pyrethroid insecticides [10].

Among the Ca^{2+}- and calmodulin-dependent enzymes, calcineurin is unique in that the established specific inhibitors, cyclosporin A and FK506, each requires a distinct protein cofactor to exert an inhibitory effect. Although the pyrethroid insecticides have been reported to inhibit calcineurin directly with no involvement of protein cofactors, the pyrethroids also were tested under conditions permissive for calcineurin inhibition by cyclosporin A and FK506.

In agreement with our results from experiments using purified enzyme, the pyrethroids exhibited no inhibitory effect on calcineurin activity in the supernatants from crude brain homogenate or in cultured cells. In contrast, potent inhibition of calcineurin by cyclosporin A and FK506 was observed in these experiments. The inability of these agents to inhibit okadaic acid-sensitive phosphatase activity expands these observations to the other major phosphatases by demonstrating that the pyrethroids do not inhibit the activity of type-1 or type-2A phosphatases.

Although it is possible that the discrepancy between the results presented herein and the previously published reports on the inhibitory effect of pyrethroids on calcineurin can be explained by differences in experimental methodology, this is unlikely. The use of polyethylene glycol-coated glass tubes and the careful avoidance of all plasticware, which has been suggested to be important for maintaining the inhibitory activity of the pyrethroids, did not alter our experimental results. In light of the fact that type II pyrethroid insecticides currently are used as calcineurin inhibitors, the results of our study illustrate the importance of careful controls in any study using these compounds to inhibit calcineurin. The belief that the pyrethroids are effective calcineurin inhibitors has resulted in their use for this purpose by numerous investigators without substantiating that calcineurin phosphatase activity was indeed inhibited [21–30]. One additional factor that must be considered in this work is the amount of ethanol introduced as vehicle for agents like pyrethroid insecticides. Because these compounds are difficult to maintain in aqueous solution, vehicle is added at concentrations that are clearly inhibitory for calcineurin. In the experiments reported here, we used a modestly inhibitory concentration of ethanol as a vehicle. Under these conditions, we did not detect any activity in the type II pyrethroid insecticides, which would indicate they have a specific interaction with calcineurin, calmodulin, or the type-1 or type-2A phosphatase.

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