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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.

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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