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SHORT COMMUNICATION

Class II Pyrethroids: Noninhibitors Calcineurin

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ABSTRACT. Type II pyrethroid insecticides have been reported to be potent inhibitors of bovine brain calcineurin (EC 3.1.3.16, Enan E and Matsumara F, *Biochem Pharmacol* **43:** 1777–1784, 1992). In concentrations up to 10^{-5} M, none of the pyrethroid insecticides used in this study caused inhibition of the calcineurin-dependent dephosphorylation of the 19-amino acid phosphopeptide derived from the regulatory subunit R-II of the cyclic adenosine 3′,5′-monophosphate (cAMP)-dependent protein kinase, which has been established as a good substrate for this enzyme. Neither did any of the compounds tested cause a shift in the inhibitory activity of okadaic acid (apparent K_i of 5 μM). The assumption that calcineurin is generally inhibited by pyrethroid insecticides is incorrect, and the interpretation of cellular experiments in which this assumption has been made must be revised. BIOCHEM PHARMACOL **54**;2:321–323, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. calcineurin; protein phosphatases; enzyme inhibitors; pyrethroids; okadaic acid.

Calcineurin (CLN or PP2B, EC 3.1.3.16) is a calcium- and calmodulin-regulated protein phosphatase that is involved in the regulation of a wide variety of biological processes. In particular, the discovery that PP2B is the biological target of both cyclosporin A (CyA)† and FK-506 in immunosuppression has enhanced interest in finding novel PP2B inhibitors. Both CyA and FK-506 must, however, form complexes with binding proteins (immunophillins) to exert their inhibitory action on PP2B. In 1992, Enan and Matsumara [1] reported that class II pyrethroids such as cypermethrin, deltamethrin, and fenvalerate were potent inhibitors of PP2B. This publication has been quoted by a number of workers who have used such pyrethroids in cellular experiments, and who have thus assumed to have inhibited PP2B activity (for some examples, see [2–6]). We have made numerous unsuccessful attempts to repeat this observation, and now report that cypermethrin, deltamethrin, and fenvalerate do not inhibit the dephosphorylation of a well-accepted phosphopeptide substrate by PP2B in a well-characterised and established assay.

MATERIALS AND METHODS

Okadaic acid was purchased from LC Services Corp. (Woburn, MA) and microcystin-LR obtained from Calbiochem (La Jolla, CA). *p*-Nitrophenyl phosphate (PNPP) was obtained from Merck (Darmstadt, Germany). The pyrethroid insecticide samples bioallethrin [(*S*)-3-allyl-2-methyl-4-oxocyclopent-2-enyl-(1*R*, 3*R*)-2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropanecarboxylate], cyfluthrin [cyano-(4-fluoro-3-phenoxyphenyl)-methyl-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate], cypermethrin [cyano-(3-phenoxyphenyl)-methyl-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate], deltamethrin [(*S*)-α-cyano-3-phenoxybenzyl-(1*R*)-*cis*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate], and fenvalerate [4-chloro-α-(1-methylethyl)phenylacetic acid-α-cyano-3-phenoxybenzyl ester] were acquired from Riedel de Haaen (Seelze, Germany). (The nomenclature for the configuration of the pyrethroids follows that proposed by Elliott M, James NF, Pulman DA, *J. Chem. Soc. Perkin Trans.* **1:** 2470–2474, 1974. See also *Pesticide Handbook*, 10th ed., Royal Society of Chemistry, 1995.) Esfenvalerat, alphamethrin, zeta-cypermethrin, and an additional sample of cypermethrin were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Bioallethrin was present as a *ca.* 1:1 mixture of diastereoisomers at the four-position with *cis*-configuration of the substituents of the cyclopropyl moiety, as deduced from ^{1}H-NMR measurements. Deltamethrin was available as a pure (1*R*) enantiomer, whose structure and configuration was confirmed by ^{1}H-NMR, MS, and optical rotation [mp 100°, $[\alpha]_D$ +8.7 (c = 0.7, acetone)]. Fenvalerate is a racemic mixture of four diastereoisomers (*RS*:*SS*:*RR*:*SR* 1:1:1:1), and cyfluthrin, cypermethrin, and zeta-cypermethrin are mixtures of diastereoisomers. Alfamethrin (also called alpha-cypermethrin) is a racemate of (*S*)-α-cyano-3-phenoxybenzyl-(1*R*)-*cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate and (*R*)-α-cyano-3-phenoxybenzyl-(1*S*)-*cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate, while esfenvalerate (also known as fenvalerateAα) is a single enantiomer with (*S*, *S*) configuration. Bovine brain PP2B was purchased from UBI (Lake Placid, NY, Lot No. 12263). PP2B phos-

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† *Abbreviations*: cAMP: cyclic adenosine 3′,5′-monophosphate; CyA, cyclosporin A; PNPP, *p*-nitrophenyl phosphate; PP2B, protein phosphatase 2B.

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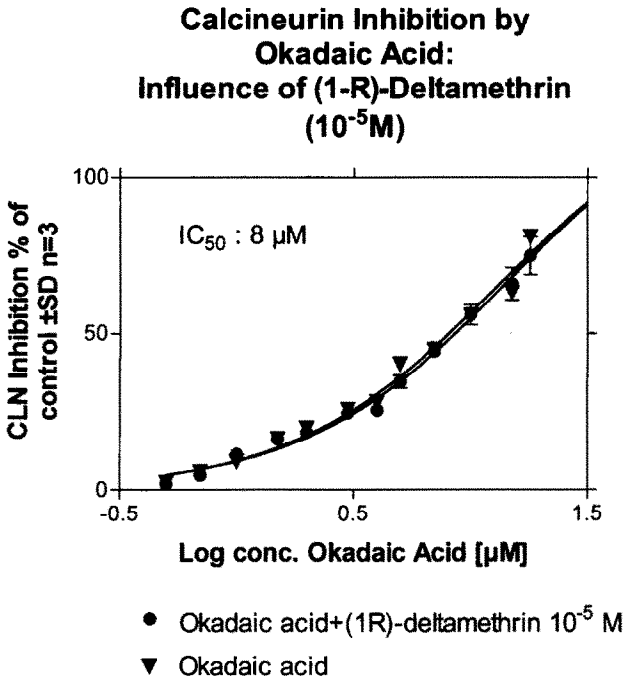

FIG. 1. Calcineurin inhibition by okadaic acid: influence of (1R)-deltamethrin (10^{-5} M). IC_{50} was calculated by curve fitting with a nonlinear regression program (Prism, GraphPad, San Diego, CA). The mean value ± SD (n = 4) of the control enzymatic activity was 5.4 nmol × ng^{-1} × min^{-1} ± 0.14.

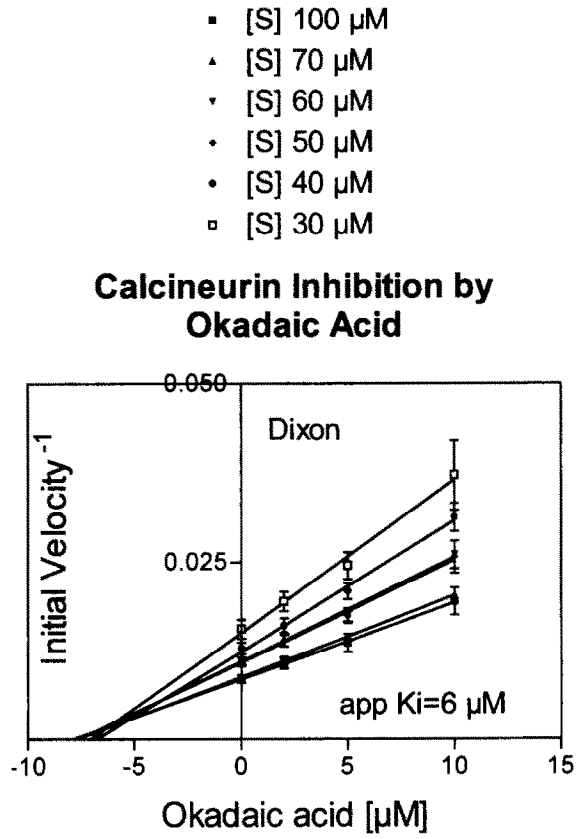

FIG. 2. Dixon plot of the inhibition of calcineurin by okadaic acid.

phatase activity was determined by the HPLC assay previously described in detail [7] by monitoring the dephosphorylation of a 19-amino acid phosphopeptide, a partial sequence of the regulatory subunit of cyclic adenosine 3′,5′-monophosphate (cAMP)-dependent protein kinase [8]. The pyrethroids were dissolved in EtOH at 10^{-2} M. These stock solutions were further diluted with the assay buffer. The final concentration of EtOH in the test solutions was 0.1% or below.

RESULTS

In initial experiments using PNPP as a substrate, we could not observe any pyrethroid-dependent PP2B inhibition. We have developed a nonradioactive HPLC assay that allows the simultaneous observation of the substrate phosphopeptide and the product dephosphorylated peptide. With the cyclosporinA/cyclophilinA complex as well as with microcystin-LR, a clear inhibition was reported with this substrate [3]. The same has now been found with okadaic acid, which had previously been shown to be an inhibitor of PP2B-dependent dephosphorylation [9]. At 50 µM substrate concentration, the IC_{50} was estimated to be 8 µM (Fig. 1). Enzyme kinetics experiments resulted in an apparent K_i of 5 µM (K_M = 60 µM), as can be deduced from the Dixon plots (Fig. 2). None of the pyrethroid insecticides had any effect on the dephosphorylation of the peptide substrate at concentrations of 10^{-8}–10^{-5} M. Additionally, (1R)-deltamethrin and esfenvalerate (10^{-5} M) were incubated with the enzyme in the presence of okadaic acid, and the inhibition of PP2B was, within experimental error, indistinguishable from that of okadaic acid alone. Figure 1 shows the inhibition curves obtained with okadaic acid and (1R)-deltamethrin.

DISCUSSION

Enan and Matsumara [1] reported that bioallethrin (10^{-9} M) inhibited the dephosphorylation of O-phospho-DL-tyrosine by PP2B to 65% but did not inhibit the dephosphorylation of PNPP. In contrast, cypermethrin and (1R)-deltamethrin (both IC_{50} *ca.* 10^{-11} M) and fenvalerate Aα (IC_{50} 10^{-8} M) were claimed to be potent inhibitors of both PNPP and O-phospho-DL-tyrosine dephosphorylation by PP2B. [Enan and Matsumara did not specify the composition of cypermethrin they used, presumably an equimolar mixture of all eight diastereoisomers; additionally, they only identified one of the three chiral centres of deltamethrin. We assume that their reference to (1R)-deltamethrin corresponds to (S)-α-cyano-3-phenoxybenzyl-(1R)-*cis*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate, and (1S)-deltamethrin to its enantiomer, rather than to another diastereoisomer.] The isomers (1S)-deltamethrin and fenvalerateBβ [i.e. (R,R)] were reported to be markedly less active. We have observed that the compounds claimed to be active inhibitors of PP2B were all unable to inhibit the

dephosphorylation of a well-characterised phosphopeptide substrate. Other workers have reported an inability to reproduce the original observations of Enan and Matsumara, and a modification of the original procedure has appeared [10]. In our hands, this modification did not result in any inhibition of PP2B. Pyrethroid insecticides may exert effects on intracellular phosphorylation processes by a variety of mechanisms, among which may be direct effects on kinases, as claimed by the same authors [11], or indirect effects due to sodium and calcium channel modulation [12]. In any case, pyrethroid insecticides cannot be considered as general and specific inhibitors of PP2B-dependent processes. There may, however, be circumstances under which these compounds interact with PP2B, but direct evidence of PP2B inhibition under the experimental conditions should be obtained.

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