Biochemical Pharmacology, Vol. 43, No. 8, pp. 1777–1784, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

SPECIFIC INHIBITION OF CALCINEURIN BY TYPE II SYNTHETIC PYRETHROID INSECTICIDES

ESSAM ENAN and FUMIO MATSUMURA*
Department of Environmental Toxicology and Toxic Substance Research and Teaching Program, University of California, Davis, CA 95616, U.S.A.

(*Received* 29 *July* 1991; *accepted* 8 *January* 1992)

Abstract—The inhibitory action of synthetic pyrethroids and some chlorinated hydrocarbon insecticides on the neural calcium–calmodulin-dependent protein phosphatase, calcineurin, was studied using one radiotracer and two colorimetric methods. It was found that all insecticidal Type II pyrethroids (cypermethrin, deltamethrin and fenvalerate) are potent inhibitors of isolated calcineurin from bovine brain. Their IC_{50} values were approximately 10^{-9} to 10^{-11} M. By contrast, neither noninsecticidal chiral isomers of these pyrethroids, neuroactive Type I pyrethroids nor neuroactive chlorinated hydrocarbon insecticides showed comparable potencies against this enzyme. To confirm the action of Type II pyrethroid *in situ*, isolated intact rat brain synaptosomes were incubated with [^{32}P]phosphoric acid and subsequently depolarized in the presence and absence of 0.1 μM deltamethrin. As expected, there was a sharp rise in protein phosphorylation due to the action of calcineurin. Deltamethrin caused a distinct delay in the dephosphorylation process. The results clearly indicate that calcineurin is specifically inhibited by Type II pyrethroids.

Pyrethroids are very potent insecticides which are now widely used [1]. Among them highly modified Type II pyrethroids are particularly known for their chemical and biological stability and the unusual *in vivo* symptoms they cause.

That DDT and pyrethroids primarily attack the sodium channel has been well established [2–4]. The latter compounds, particularly those classified as Type II, are known to cause prolonged Na^+ current [5, 6], indicating their effects on both inactivation and tail current processes of the gate operation. However, it has been noted by several groups of scientists [3, 7, 8] that there are roughly two different types of pyrethroid actions, and that the newly developed compounds (termed Type II pyrethroids) affect animals in a way different from that of the older compounds (Type I pyrethroids). One of the most recognizable effects of Type II compounds is profound stimulation of neurotransmitter release at the synapse [4, 9–11]. A part of such stimulation is caused by their effect on the sodium channel itself [10, 12], but there is still a significant part of the stimulation in the rat brain and the squid synapses that cannot be abolished by treatment with tetrodotoxin (0.1 to 1 μM) [13, 14]. As to the cause of this portion of the Type II pyrethroid action, Brooks and Clark [11] have shown that it does not involve the initial depolarization process, since the phenomenon is not observed unless the transmitter release process is first triggered using high K^+, veratridine or high Ca^{2+} (initiated by A23187). Such initial stimulatory actions may be followed, at least in some instances, by synaptic blockage. Electron microscopic observations have revealed that in treated synapses the synaptic vesicles move toward the synaptic gap upon depolarization, and even after cessation of depolarizing signals they remain piled up at the terminal region of the presynaptic complex [15].

It has been clearly established that protein phosphorylation–dephosphorylation processes play vital roles in synaptic transmission [16, 17]. Upon depolarization by high K^+ or veratridine (or by any other depolarizing treatments, for that matter), the rate of protein phosphorylation within the presynaptic terminal promptly rises as originally reported by Schulman and Greengard [18]. After reaching the peak (in about 15 sec, depending on the protein), relatively slow dephosphorylation processes take over which last for about 3–5 min [19]. Such phosphorylation–dephosphorylation activities are very sensitive to the changes in the external concentration of Ca^{2+}. Recently, we have shown that deltamethrin, a typical Type II pyrethroid, causes a significant and persistent rise in protein phosphorylation in intact synaptosomes from the brain [20] which appears to be causally related to its stimulatory action on the neurotransmitter releasing process. In the current work we have further studied the biochemical cause of this phenomenon and found that Type II pyrethroids produce a group-specific inhibition on an important phosphatase, calcineurin.

EXPERIMENTAL PROCEDURES

Materials. [γ-^{32}P]ATP (~3000 Ci/mmol) and [^{32}P]-H_3PO_4 (200 mCi/mmol) were purchased from Amersham. Bovine brain calmodulin (CaM†),

* Corresponding author: Dr. Fumio Matsumura, Department of Environmental Toxicology and Toxic Substance Research & Teaching Program, University of California, Davis, CA 95616. Tel. (916) 752-4251 or (916) 752-3394; FAX (916) 752-5300.

† Abbreviations: CaM, calmodulin; pNPP, *p*-nitrophenylphosphate; MOPS, (3-[*N*-morpholino] propanesulfonic acid) free acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; and SDS, sodium dodecyl sulfate.

bovine brain calcineurin (500 units, 3000 units/mg protein), *p*-nitrophenylphosphate (pNPP), MOPS (3-[*N*-morpholino]propanesulfonic acid) free acid, *O*-phospho-DL-tyrosine, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), malachite green hydrochloride, ammonium molybdate, Triton X-100, protein kinase-catalytic subunit (500 units, 0.023 mg protein), histone from calf thymus (Type II-S) and all other chemicals were purchased from the Sigma Chemical Co. Phosphocellulose paper was purchased from Whatman International Ltd., Maidstone, England (P-18 type). Young male Sprague–Dawley rats were used.

Assay of calcium–calmodulin-dependent protein-phosphatase (calcineurin) activity. Calcineurin activity was measured using either colorimetric methods or radiotracer techniques. Calorimetric assays for *p*-nitrophenol or inorganic phosphate were carried out according to the methods of Martin *et al.* [21], with slight modifications as follows.

Assay for p-*nitrophenol.* The activity of partially purified, bovine brain calcineurin on the artificial substrate *p*-nitrophenylphosphate was measured by the release of the *p*-nitrophenolate anion. Using this method, 1–1000 nM concentrations of insecticides were used against calcineurin activity. Each insecticide was incubated with calcineurin (0.5 μg protein) for 10 min at 30° in a reaction mixture containing 25 mM MOPS buffer (pH 7.0), 0.3 mM $CaCl_2$, and 25 nM CaM. Thereafter, 7 mM *p*-nitrophenylphosphate was added to each tube, giving a final volume of 100 μL, and incubated for an additional 30 min at 30°, and the absorbance was read at 410 nm. The reaction mixture was diluted to 1.0 mL with 0.1 M glycine (pH 9.5). IC_{50} Values were determined for the active and inactive forms of deltamethrin and fenvalerate.

Assay for inorganic phosphate. The release of P_i was assayed when *O*-phospho-DL-tyrosine was used as a substrate for calcineurin. Several concentrations of each insecticide were incubated with calcineurin for 10 min at 30° under the same conditions as above. Tyrosine phosphate (1 mM, final concentration) was added to each tube giving a final volume of 100 μL, and incubated for an additional 40 min at 30°. The reaction mixtures were diluted to 900 μL with the malachite green reagent [21]. The samples were mixed, and the absorbance was measured at 660 nm after 10 min. IC_{50} Values were determined for all the insecticides that gave over 50% inhibition at approximately 1 nM in a preliminary study. The activity of the enzyme was expressed as the micromolar concentration of P_i released in 100 μL volume per 40 min.

Radiotracer techniques. The purpose of this assay was to measure the dephosphorylation rate of the phosphorylated (^{32}P) protein substrates (either artificial or naturally occurring). The different methods we used are described below.

Preparation of [^{32}P]*phosphohistone.* Histone Type II-S (30 mg) was dissolved in 5 mL of HEPES buffer at pH 7.4 (50 mM HEPES, 10 mM $MgCl_2$ and 1 mM dithiothreitol). To this preparation, 10 μg of cAMP-dependent protein kinase (catalytic subunit), 5 μM ATP and 30 μCi of [γ-^{32}P]ATP were added. This mixture was incubated at 25° for 1 hr in a shaking water bath, followed by overnight dialysis (mol. wt cutoff of 6000–8000) in 2 L of 5 mM HEPES buffer (pH 7.4) at 4°. The labeled phosphohistone was divided into 250-μL aliquots and frozen at −80° until used.

In vitro *(cell-free system) studies of rat brain calcineurin.* The lysed rat brain synaptosomal membrane fraction was used as a source of rat brain calcineurin [22]. A 100 μg protein portion of this preparation was incubated for 10 min at 30° with 10^{-7} M deltamethrin (dissolved in 1 μL ethanol) in a reaction mixture containing 50 mM HEPES buffer (pH 7.4), 0.3 mM $CaCl_2$ and 25 nM calmodulin in a total volume of 100 μL. The reaction was initiated by adding 100 μL of [^{32}P]phosphohistone, and the mixture was incubated for different time intervals of 0, 1, 3, 5, 10 and 30 min at 30°. The control tubes received the same volume of ethanol (1% of the total volume of the reaction) as the treated tubes. After an appropriate incubation period, each tube received 50 μL of the stop solution [0.3 M EDTA (disodium salt) containing 2 mg/mL bovine serum albumin] followed immediately by the addition of 1 mL of ice-cold 20% trichloroacetic acid (TCA) [23]. After 10 min at 4°, the tubes were centrifuged at 1000 *g* for 2 min. The precipitate was redissolved in 100 μL of 0.1 N NaOH, and reprecipitated by adding 1 mL of 20% TCA. This process was repeated two times. The final precipitate was redissolved in 200 μL of 0.1 N NaOH and transferred into a scintillation vial containing 5 mL of liquid scintillation solution for radioassay to assess the amount of [^{32}P]-phosphohistone that remained.

Effect of deltamethrin on the phosphorylation–dephosphorylation of endogenous phosphorylated lysed membrane proteins. A 100 μg protein sample of the lysed membranes of rat brain synaptosomes was incubated with 10^{-7} M deltamethrin for 10 min at 30° exactly as described above. The control tubes received the same volume of ethanol. To each tube, 0.04 μCi of [γ-^{32}P]ATP was added and incubated at 30° for time intervals of 0, 15, 30, 60 or 600 sec. The phosphorylation–dephosphorylation system was stopped by the addition of 4X-SDS treatment buffer [20]. The samples were mixed and boiled in a water bath for 2 min and then cooled. The entire volume of the reaction product in each tube was transferred to the well of a 7% sodium dodecyl sulfate (SDS)–polyacrylamide slab gel [24] and electrophoresed as described by Enan and Matsumura [20]. The dried gel was exposed to X-ray film for 2 weeks before developing. The autoradiograms obtained were scanned using a densitometer connected to an HP 3396 A Integrator to determine the level of phosphorylation on each protein band.

In situ *effect of deltamethrin on calcineurin.* The fresh rat brain synaptosomes (300 μg protein) were phosphorylated with 0.2 μCi of [^{32}P]H_3PO_4 as described previously [25]. After 30 min of incubation at 37°, 10^{-7} M deltamethrin in 1 μL/mL of ethanol was added to the synaptosomes (control received the same volume of ethanol). The incubation continued for an additional 15 min at 37°. A 50-μL aliquot from either treated or control tubes was transferred into another test tube containing 50 μL of the depolarizing buffer (high K^+, 62 mM) [20]

Table 1. Effects of a 10^{-9} M concentration of different insecticides on bovine brain calcineurin activity using *p*-nitrophenylphosphate as substrate (7 mM)

Insecticides	*p*-Nitrophenol* (μM/30 min)	% Calcineurin activity remaining*
Control	7.50 ± 0.18†	100
Type I pyrethroids		
Pyrethrins	7.01 ± 0.17	93.5
Allethrin	7.50 ± 0.16	100
Resmethrin	7.25 ± 0.14	96.7
cis-Permethrin	7.25 ± 0.16	96.7
trans-Permethrin	7.50 ± 0.10	100
Type II pyrethroids		
Cypermethrin	3.50 ± 0.10‡	46.7
Fenvalerate (Aα)	4.35 ± 0.12‡	58.0
Fenvalerate (Bβ)	6.53 ± 0.09	87.0
Deltamethrin (1*R*)	4.25 ± 0.07‡	56.7
Deltamethrin (1*S*)	7.13 ± 0.18	95.0
Chlorinated hydrocarbons		
DDT	6.75 ± 0.17	90.0
Heptachlor epoxide	6.50 ± 0.16	86.7

* All the values are averages for four independent experiments.
† Mean ± SD.
‡ Significantly different from the control value at $P \leq 0.01$.

Table 2. Effect of a 10^{-9} M concentration of some insecticides on bovine brain calcineurin activity using 1 mM *O*-phospho-DL-tyrosine as substrate

Insecticides	Calcineurin activity*	
	P_i (μM)/40 min	% Calcineurin activity remaining
Control	6.6 ± 0.9†	100
Type I pyrethroids		
Pyrethrins	5.4 ± 0.6	82
Resmethrin	6.2 ± 1.0	94
cis-Permethrin	5.8 ± 1.2	88
trans-Permethrin	5.6 ± 1.6	85
S-Bioallethrin	4.5 ± 2.2	68
Bioallethrin	4.3 ± 1.2	65
Tetramethrin	5.6 ± 1.4	85
Type II pyrethroids		
Cypermethrin	1.3 ± 0.2‡	20
Fenvalerate (Aα)	4.1 ± 0.9‡	62
Fenvalerate (Bβ)	5.8 ± 1.8	88
Deltamethrin (1*R*)	2.9 ± 0.8‡	44
Deltamethrin (1*S*)	5.0 ± 1.6	76
Chlorinated hydrocarbons		
DDT	4.7 ± 1.1	71
Heptacholar epoxide	5.7 ± 1.6	86

* All the values are averages of four independent experiments.
† Mean ± SD.
‡ Significantly different from the control value at $P \leq 0.01$.

and incubated for 60 sec at 37°. The depolarization reaction was stopped by diluting the system ten times with 50 mM HEPES buffer (pH 7.4) containing 25 nM calmodulin and 0.3 mM $CaCl_2$. This mixture was homogenized and the phosphatase reaction was assessed by further incubation for 0, 30, 300 and 600 sec. After incubation, a 20-μL aliquot was taken out and spotted onto a piece of phosphocellulose paper (2 cm × 1 cm) which was allowed to dry at room temperature for 6 min. The papers were rinsed in 3 mL of 85 mM H_3PO_4 twice (3 min each time). After drying, the papers were transferred into scintillation vials for radiocounting.

A parallel experiment was carried out with fresh

rat brain synaptosomes but without $[^{32}P]H_3PO_4$. In this experiment deltamethrin (10^{-7} M final concentration) was incubated first with intact rat brain synaptosomes (1 mg protein) for 15 min at 37°. The reaction product was diluted 10-fold (5 mL) with 50 mM HEPES buffer (pH 7.4) and sonicated for 5 sec twice. The sonicated synaptosomes were centrifuged at 20,000 *g* for 30 min at 4° using a Sorvall high speed cooling centrifuge. A 50-μg protein portion of the supernatant was incubated with 25 nM calmodulin, 0.3 mM $CaCl_2$ and 100 μg of ^{32}P-labeled phosphohistone in a total volume of 100 μL for 0, 30, 60, 180, 300 and 600 sec at 30°. After incubation, 20-μL aliquots were taken out and spotted onto phosphocellulose paper (2 cm × 1 cm). These papers were treated exactly as described above and radioassayed.

For electrophoresis studies, the same experiment was repeated using a 10^{-6} M concentration of other insecticides. The supernatant from the sonicated synaptosomes was incubated with calmodulin, $CaCl_2$ and $[^{32}P]$phosphohistone in the presence and absence of insecticides. The reaction product was centrifuged at 100 *g* for 3 min, and 100 μL of the supernatant was mixed with 50 μL of 4X-SDS treatment buffer [20]. After boiling for 2 min, the product was transferred to the well of a 7% SDS–polyacrylamide slab gel and electrophoresed as described above. The dried gel was scanned using a computerized radioscanner (Ambis Systems Inc., San Diego, CA).

RESULTS

Effect of synthetic pyrethroid insecticides on isolated bovine brain calcineurin. The effects of a 10^{-9} M concentration of Type I and Type II pyrethroids on calcium/calmodulin-dependent protein phosphatase activity were compared colorimetrically using *p*-nitrophenylphosphate as a substrate (Table 1). Inhibition of calcineurin by active forms of Type II synthetic pyrethroids, compared to the control, was statistically significant ($P \leq 0.01$, Student's *t*-test). By contrast, none of Type I pyrethroids, DDT or heptachlor epoxide exhibited a significant inhibitory action against the enzymatic hydrolysis of *p*-nitrophenylphosphate. The concentration responses of calcineurin toward active and nonactive optical isomers of deltamethrin and fenvalerate were studied using the same substrate (Fig. 1). The IC_{50} value was found to be approximately 3×10^{-9} M for fenvalerate (Aα active form), but for inactive isomers the values were higher than 10^{-5} M. The response of calcineurin to the active isomer of deltamethrin (1*R*) appeared to be biphasic, but the overall I_{50} was about 3×10^{-11} M, assuming a monophasic relationship.

Table 2 contains measured values for calcineurin responses to pyrethroids (preincubated for 10 min at 30° with Type I or Type II pyrethroids) using dephosphorylation of *O*-phospho-DL-tyrosine as a parameter. Again there was a distinct group difference between Type I and Type II pyrethroids with regard to their inhibitory actions on this enzyme. One minor difference between the two assay methods is that bioallethrin (10^{-9} M), a type I pyrethroid, markedly reduced (to 65%) calcineurin activity only when tyrosine phosphate was the substrate. However,

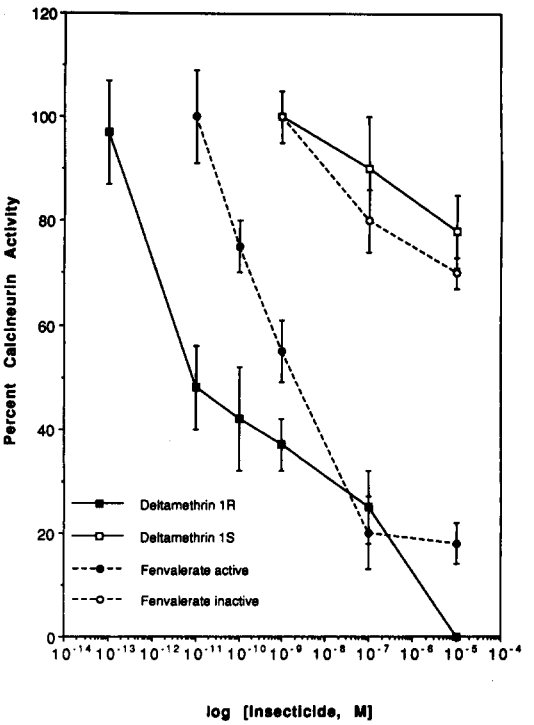

Fig. 1. Concentration–inhibitory activity relationship between Type II pyrethroids and calcineurin. Different concentrations of the active and nonactive forms of these insecticides were incubated for 10 min with purified bovine brain calcineurin, and the activity of this enzyme was assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenolphosphate through spectroscopic means. Data are expressed as relative percentages to control values (=100) which were obtained by preincubating with the same volume of ethanol. Each point is the mean ± SD of four independent experiments, each determined in duplicate. The control value (=100) was 7.5 ± 0.18 μM *p*-nitrophenol/30 min.

the IC_{50} value for bioallethrin was much higher than those of Type II synthetic pyrethroids (i.e. $>10^{-5}$ M for bioallethrin as compared to 10^{-11} M for cypermethrin and deltamethrin 1*R* and 10^{-8} M for fenvalerate Aα) (Fig. 2), indicating a profound potency difference between these two groups of pyrethroids. In addition, the inhibitory powers of DDT and heptachlor epoxide were found to be very low (Table 2). These *in vitro* data led us to postulate that this difference in enzyme inhibition could be a good probe for differentiating the action patterns of Type II synthetic pyrethroids from other neuroactive insecticides including Type I pyrethroids and organochlorine insecticides. To support this hypothesis, however, it is necessary to measure the inhibitory potencies of these insecticides within an intact cellular system containing an active calcium/CaM-dependent protein phosphorylation–dephosphorylation system. Furthermore, it is necessary to show that such inhibitory actions result in actual changes in phosphorylation levels of vital proteins whose functions are known under relevant physio-

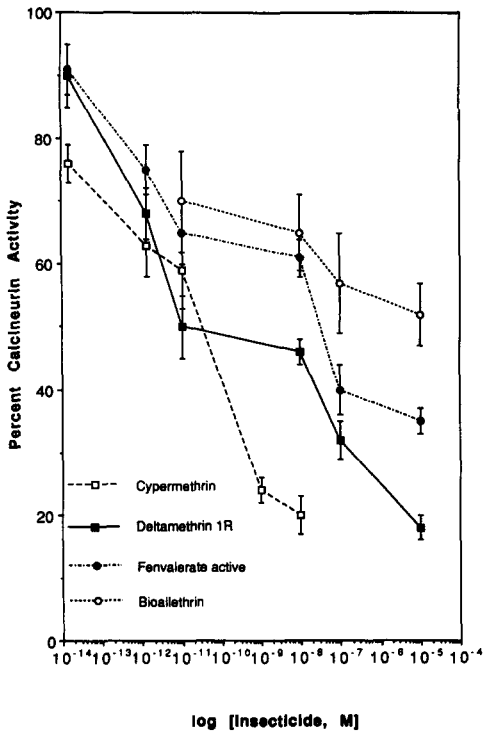

Fig. 2. Concentration–inhibitory activity relationship between certain pyrethroids, and calcineurin. Experimental conditions were the same as those described in the legend of Fig. 1 except for the use of *O*-phospho-DL-tyrosine as a substrate. Released P_i was measured by the malachite green-colorimetric assay as described in Experimental Procedures. Data points are the means ± SD of four independent experiments, each determined in triplicate. The control value (=100) was 6.6 ± 0.9 μM P_i released/40 min.

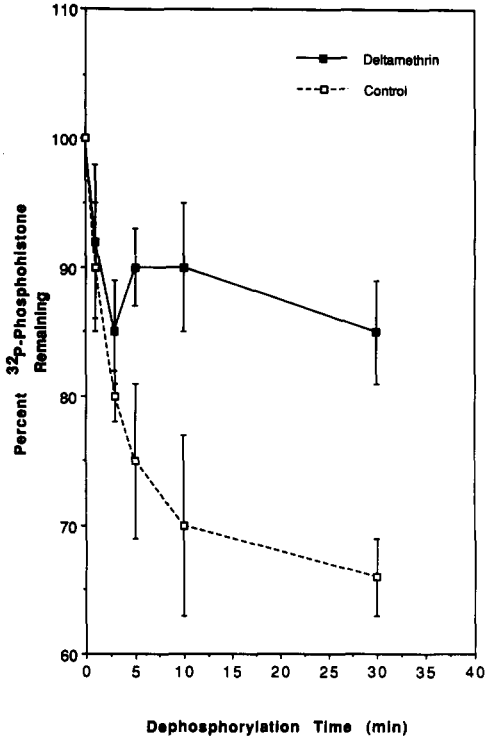

Fig. 3. Dephosphorylation of [^{32}P]phosphohistone at different time intervals. Protein (100 μg from the synaptosomal lysed membrane was incubated with 10^{-7} M deltamethrin for 10 min prior to the addition of 100 μg of [^{32}P]phosphohistone. The reaction was stopped after different time intervals (0–30 min) as described in Experimental Procedures. The remaining labeled phosphohistone in the precipitate was counted. Each point is the mean ± SD of five independent experiments, each determined in triplicate.

logical conditions. For this purpose, we selected rat brain synaptosomes for further studies.

Dephosphorylation of exogenous phosphorylated substrates by rat brain synaptosomal calcineurin. To assess the rat brain synaptosomal dephosphorylation activity [^{32}P]phosphohistone was incubated with deltamethrin-pretreated lysed synaptosomal membranes for various time periods (0–30 min) (Fig. 3). The remaining amount of [^{32}P]phosphohistone was determined by the TCA precipitation method [23]. There was an initial rapid release of $^{32}P_i$ which corresponds to the release of noncovalently bound $^{32}P_i$. This was followed by a slow but steady increase in the amount of $^{32}P_i$ release from ^{32}P-labeled histone which is likely to be due to the action of phosphatase.

Dephosphorylation of endogenous phosphorylated proteins. ^{32}P-Labeling of synaptosomal membranes and SDS-polyacrylamide slab gel electrophoresis techniques were employed to study the changes of intrasynaptosomal calcium/CaM-dependent protein kinase-phosphatase levels under the influence of 10^{-7} M deltamethrin. The lysed synaptosomal membranes were incubated with deltamethrin for 10 min at 30°, and then with [γ-^{32}P]ATP (0.04 μCi) for 15–600 sec. The reactions were stopped using SDS and the resulting ^{32}P-labeled phosphoproteins were analyzed on electrophoresis. The resulting autoradiograms indicated that, in all cases, there was an initial increase in phosphorylation due mostly to calcium/calmodulin-dependent protein kinase activity. After a 10-min incubation period, however, the level of ^{32}P-labeled protein was reduced in all tests because of the action of endogenous phosphatases. The rate of decline was faster in the control than in the deltamethrin-pretreated preparation (Fig. 4).

In situ *effect of deltamethrin on calcineurin activity.* In this *in situ* experiment, we studied the action of deltamethrine and other insecticides on the calcium/CaM-dependent protein phosphorylation–dephosphorylation process using rat brain labeled with [^{32}P]H_3PO_4. As shown in Fig. 5, the initial dephosphorylation rate of synaptosomal proteins prelabeled with [^{32}P]H_3PO_4 was much faster in the control during the first 300 sec in the presence of Ca^{2+} and calmodulin than the rate of the deltamethrin-treated synaptosomes. The same pattern of deltamethrin inhibition of the rat brain phos-

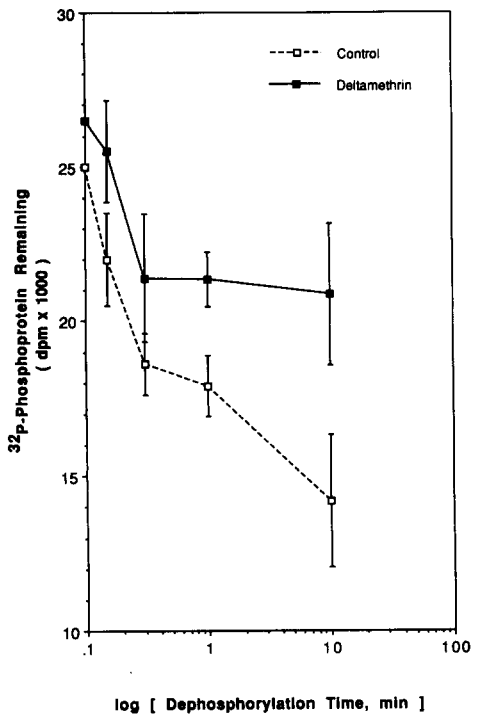

Fig. 4. Action of deltamethrin (10^{-7} M) on the phosphorylation–dephosphorylation of endogenous lysed membrane proteins from rat brain synaptosomes. Deltamethrin-preincubated lysed synaptic membranes were phosphorylated with 0.04 μCi [γ-^{32}P]ATP. The reaction was stopped at different time intervals (0–600 sec) (see Experimental Procedures). The samples were electrophoresed using a one-dimensional SDS-polyacrylamide gel and autoradiographed. The X-ray was scanned using a computerized image scanner (Ambis), and the data were plotted as means ± SD for the results of three independent experiments.

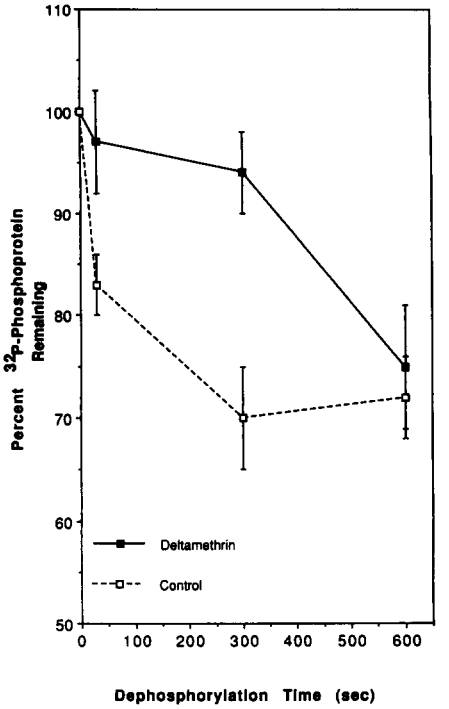

Fig. 5. *In situ* effect of deltamethrin on rat brain synaptosomal calcineurin. Intact rat brain synaptosomes were phosphorylated with 0.2 mCi of [^{32}P]H_3PO_4 for 45 min with the last 15 min in the presence and absence of 10^{-7} M deltamethrin. The synaptosomes were depolarized for 60 sec and diluted and homogenized immediately using HEPES buffer containing CaM and $CaCl_2$. The reaction product was spotted onto phosphocellulose paper for detection of [^{32}P]phosphoproteins remaining. The data are the means ± SD of five independent experiments, each determined in triplicate.

phatase was observed when [^{32}P]phosphohistone was used as a substrate in place of the endogenous [^{32}P]phosphoproteins (Fig. 6). These observations indicate that the source of [^{32}P]phosphoproteins is not important in observing the inhibitory power of deltamethrin (10^{-7} M) on the calcium/CaM-dependent protein phosphatase activity in the rat brain synapse.

Finally, selected synthetic pyrethroids and DDT were incubated with fresh rat brain synaptosomes for 15 min at 37°, and the dephosphorylation rate of [^{32}P]phosphohistone was studied as above using 7% SDS–polyacrylamide slab gel electrophoresis. The data in Fig. 7 demonstrate that Type II synthetic pyrethroids were good inhibitors of calcineurin, whereas Type I synthetic pyrethroid (allethrin) and DDT had no inhibitory power on this enzyme.

DISCUSSION

The most compelling result of these experiments is that inhibition of calcineurin appears specific to Type II pyrethroids. Type I pyrethroids, even those chemically similar to Type II (e.g. permethrins), did not affect this enzyme significantly, nor did some neuroactive chlorinated hydrocarbon insecticides. Moreover, the sensitivity of calcineurin toward Type II pyrethroids was limited to only those optical and geometric isomers that are known to be insecticidal. The difference in potencies between insecticidal and noninsecticidal isomers within the given Type II pyrethroid in this regard was enormous. Thus, there was a strong correlation between the order of potency to inhibit calcineurin and insecticidal activities among these pyrethroids tested.

These observations are consistent with results from other studies on the toxicity of deltamethrin in rat brain synaptosomes. It was reported previously that 10^{-9} M was the lowest effective concentration that caused a significant stimulation of neurotransmitter release [11, 20]. In the current study, a significant increase in protein phosphorylation as a result of calcineurin inhibition in intact synaptosomes was also observed at the 10^{-8} to 10^{-10} M range of deltamethrin.

As previously reported [26], calcineurin is composed of two subunits, 61 and 15 kD_a proteins.

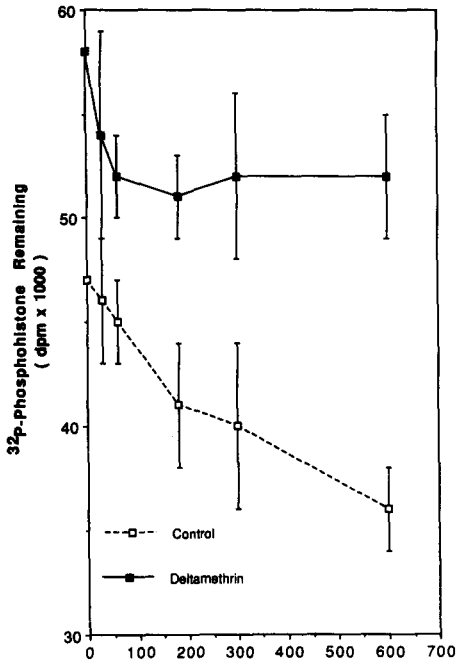

Fig. 6. Dephosphorylation of [^{32}P]phosphohistone by using preincubated synaptosomal calcineurin from rat brain. The experimental conditions were exactly as given for Fig. 5, except that the synaptosomes were not ^{32}P-phosphorylated, but after isolation of synaptosomes they were immediately incubated for 10 min with 10^{-7} M deltamethrin before dilution and homogenization with HEPES buffer without CaM or $CaCl_2$. Then, the lysed membrane was separated (see Experimental Procedures) and 100 μg protein of this preparation was incubated with 100 μg of [^{32}P]-phosphohistone. Aliquots of the reaction products were analyzed on phosphocellulose papers as before. The data are the means ± SD of five independent experiments, each determined in triplicate.

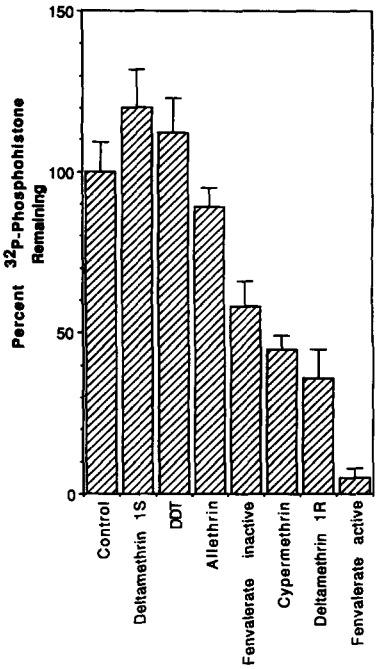

Fig. 7. Effects of different insecticides on synaptosomal calcineurin from rat brain. The synaptosomes pretreated with a 10^{-6} M concentration of the test insecticides, as shown in Fig. 5, were diluted and homogenized, and the lysed membrane was separated as described before. A 100 μg portion of this membrane was incubated for 60 min with CaM, $CaCl_2$ and 100 μg [^{32}P]phosphohistone. The incubated mixture was centrifuged and 100 μL of the supernatant was mixed with 50 μL of 4X-SDS treatment buffer and electrophoresed (see Fig. 4 caption). Fenvalerate active used was Aα and inactive was Bβ. Values are means ± SD of three experiments.

The former subunit is known to interact with calmodulin in a Ca^{2+}-dependent fashion and the latter to directly bind with Ca^{2+}. The biphasic response of calcineurin to 1*R*-deltamethrin (Fig. 1) could be related to its separate action on each subunit. Also at very high concentrations (>10^{-5} M) 1*R*-deltamethrin is known to inhibit the Ca^{2+} binding capacity of calmodulin [27].

The toxicological meaning of this inhibitory interaction is completed by the vast roles of phosphatases, which participate in many diverse cellular functions, including signal transduction pathways [28]. Therefore, in this discussion only the events related to synaptic transmitter releasing processes will be considered.

The functional importance of depolarization-induced protein phosphorylation and dephosphorylation processes in the transmitter-releasing processes has been well established [13, 19, 25]. While there could be some other minor and specific phosphatases in the synapse, calcineurin is the most dominant and wide spectrum Ca^{2+}–calmodulin-stimulated phosphatase [28]. It is known to be the main force in bringing the transmission-induced changes back to the resting state. Therefore, the expected consequence of inhibition of calcineurin is sustained neurotransmitter release and eventual blockage. Failure to return phosphoproteins to their dephosphorylated state (i.e. resting) renders them unable to respond to the next stimulus. For example, we have reported previously that one of the proteins that is phosphorylated upon stimulation by deltamethrin is synapsin I [20]. It is actually dephosphosynapsin I that is functionally active, preventing synaptic vesicles from moving toward the synaptic terminal. Thus, the failure of dephosphorylating this protein after the release of synaptic vesicular contents would not allow the synapses to terminate the discharging processes.

In conclusion, this calcium/calmodulin-dependent protein phosphatase system appears to be highly sensitive toward deltamethrin and other Type II pyrethroid insecticides. The specific inhibition of calcineurin may provide a biochemical basis for understanding the increased and prolonged neurotransmitter release in the CNS due to the basic

difference in toxic actions of Type I and Type II pyrethroids.

Acknowledgements—This work was supported by the Toxic Substances Research and Teaching Program, and Agricultural Experiment Station, University of California, Davis, and Research Grant ESO1963 from the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

REFERENCES

1. Elliott M (Ed.), *Synthetic Pyrethroids*, American Chemical Society Symposium Services No. 42, pp. 1–229. American Chemical Society, Washington, DC, 1977.
2. Narahashi T, Mechanisms of action of pyrethroids on the sodium and calcium channel gating. In: *Neuropharmacology and Pesticide Action* (Eds. Ford MG, Lunt GG, Reay RC and Usherwood PNR), pp. 36–60. Ellis Horwood, Chichester, England, 1986.
3. Staatz CG, Bloom AS and Lech JJ, A pharmacologic study of pyrethroid neurotoxicity in mice. *Pestic Biochem Physiol* **17**: 287–292, 1982.
4. Cremer JE, The influence in mammals of the pyrethroid insecticides. In: *Developments in the Science and Practice* (Eds. Hayes AW, Schnell RC and Miya TS), pp. 61–72. Elsevier Science, New York, 1983.
5. Lund AE and Narahashi T, Kinetics of sodium channel modifications as the basis of pyrethroids and DDT analogs. *Pestic Biochem Physiol* **20**: 203–206, 1983.
6. Vijverberg HPM and de Weille JR, The interaction of pyrethroids with voltage-dependent Na channels. *Neurotoxicity* **6**: 23–30, 1985.
7. Gammon DW, Brown MA and Casida JE, Two classes of pyrethroid action in the cockroach. *Pestic Biochem Physiol* **15**: 181–191, 1981.
8. Scott JG and Matsumura F, Evidence of two types of toxic actions of pyrethroids on susceptible and DDT-resistant German cockroaches. *Pestic Biochem Physiol* **19**: 141–150, 1983.
9. Salgado VL, Irving SN and Miller TA, The importance of nerve terminal depolarization in pyrethroid poisoning of insects. *Pestic Biochem Physiol* **20**: 169–175, 1983.
10. Berlin JR, Akera T, Brody TM and Matsumura F, The inotropic effects of synthetic pyrethroid decamethrin on isolated guinea pig atrial muscle. *Eur J Pharmacol* **98**: 313–322, 1984.
11. Brooks MW and Clark JM, Enhancement of norepinephrine release from rat brain synaptosomes by alpha-cyano pyrethroids. *Pestic Biochem Physiol* **28**: 127–139, 1987.
12. Nicholson RA, Wilson RG, Potter C and Black H, Pyrethroid- and DDT-evoked release of GABA from the nervous system *in vitro*. In: *Pesticide Chemistry* (Eds. Miyamoto J and Kearney PC), Vol. 3, pp. 75–78. Pergamon Press, Elmsford, NY, 1983.
13. Clark JM and Brooks MW, Neurotoxicology of pyrethroids: Single or multiple mechanisms of action? *Environ Toxicol Chem* **8**: 361–372, 1989.
14. Matsumura F, Deltamethrin-induced changes in choline transport and phosphorylation activities in synaptosomes from the optic lobe of squid *Loligo peali*. *Comp Biochem Physiol* **89C**: 179–190, 1988.
15. Seabrook GR, Duce IR and Irving SN, Block of neuromuscular transmission in housefly larvae by cypermethrin; Electrophysiological and ultrastructural correlates. *Pestic Biochem Physiol* **32**: 232–239, 1988.
16. Nestler EJ and Greengard P, *Protein Phosphorylation in the Nervous System*, pp. 382–390. John Wiley, New York, 1984.
17. Brown MD, Huganir R and Greengard P, Protein phosphorylation and neuronal function. *J Neurochem* **45**: 11–23, 1985.
18. Schulman H and Greengard P, Stimulation of brain membrane protein phosphorylation by calcium and endogenous heat-stable protein. *Nature* **271**: 478–479, 1978.
19. Robinson PJ and Dunkley PR, Depolarisation-dependent protein phosphorylation and dephosphorylation in rat cortical synaptosomes is modulated by calcium. *J Neurochem* **44**: 338–348, 1985.
20. Enan EE and Matsumura F, Stimulation of protein phosphorylation in intact rat brain synaptosomes by a pyrethroid insecticide, deltamethrin. *Pestic Biochem Physiol* **39**: 182–195, 1991.
21. Martin B, Pallen CJ, Wang JH and Graves DJ, Use of fluorinated tyrosine phosphates to probe the substrate specificity of the low molecular weight phosphatase activity of calcineurin. *J Biol Chem* **260**: 14932–14937, 1985.
22. Hajos F, An improved method for the preparation of synaptosomal fractions in high purity. *Brain Res* **93**: 485–488, 1975.
23. Kennedy MB, McGuinness T and Greengard P, A calcium–calmodulin dependent protein kinase from mammalian brain that phosphorylates synapsin I: Partial purification and characterization. *J Neurosci* **3**: 818–831, 1983.
24. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–681, 1970.
25. Robinson PJ and Dunkley PR, Depolarization-dependent protein phosphorylation in rat cortical synaptosomes: Factors determining the magnitude of the response. *J Neurochem* **41**: 909–918, 1983.
26. Klee CB, Crouch TH and Krinks MH, Calcineurin: A calcium- and calmodulin-binding protein of the nervous system. *Proc Natl Acad Sci USA* **76**: 6270–7273, 1979.
27. Rashatwar SS and Matsumura F, Interaction of DDT and pyrethroids with calmodulin and its significance in expression of enzyme activities of phosphodiesterase. *Biochem Pharmacol* **34**: 1689–1694, 1985.
28. Cohen P, The structure and regulation of protein phosphatase. *Annu Rev Biochem* **58**: 453–508, 1989.