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ACTIVATION OF PHOSPHOINOSITIDE/PROTEIN KINASE C PATHWAY IN RAT BRAIN TISSUE BY PYRETHROIDS

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Abstract—We have investigated the effects of a type II pyrethroid insecticide, deltamethrin, on changes in the protein phosphorylation pattern associated with neurotransmitter release in rat brain synaptosomal preparations. Deltamethrin was found to stimulate directly the activity of the protein kinase C/phosphoinositide pathway at very low concentrations. This action resulted in an increase in the intracellular concentration of inositol 1,4,5-triphosphate (IP_3) and free calcium, as well as an increase in overall and specific protein phosphorylation within the synapse. Particularly noticeable was the deltamethrin-induced increase in phosphorylation on two very acidic proteins (87 and 48 kDa proteins) and one basic 38 kDa protein. These results are consistent with those of a previously reported study in which deltamethrin caused an increase in neurotransmitter release which was accompanied by increased intrasynaptosomal free Ca^{2+} levels and protein phosphorylation activities. Together all these observations support the view that calcium-sensitive proteins involving synaptic transmission are the major action targets of type II pyrethroids.

Pyrethroids are probably the most potent insecticides ever developed [1]. Despite their widespread use today, for a variety of pest control measures, the mechanisms by which they cause severe neurotoxic effects are not well understood. This is particularly true with the newly developed pyrethroids, designated as type II, which are known to cause unusual toxic symptoms (such as choreoathetosis) [2, 3] and behavioral changes [4] that are quite different from those caused by classic pyrethroids. Recent reports [5, 6] have indicated that in a rat brain synaptosomal preparation, deltamethrin, one of the most potent type II pyrethroids, causes tremendous stimulation of neurotransmitter release at concentrations as low as 10^{-11} M. In addition, we have shown recently [7] that deltamethrin causes a rise in protein phosphorylation within the synaptic process which is accompanied by an increase in intracellular Ca^{2+} concentration. Since such an increase of Ca^{2+} was also observed, even when the external Ca^{2+} was totally replaced by Ba^{2+}, we have concluded that deltamethrin is probably able to stimulate the release of Ca^{2+} from internal storage sites. This stimulatory action of deltamethrin on neural processes could only be recognized following depolarization of the synaptosomes.

In view of the toxicological significance of such an action, we have extended this line of investigation into the biochemical cause(s) for stimulation of synaptic activities by studying the action of deltamethrin on protein kinase C/phosphoinositide pathways. It is well known that protein kinase C (PKC)† can modulate many neuronal processes [8]. These include neurotransmitter release [9–11], signal transduction [12], ion channels [13] and synaptic plasticity [14].

In the present work, we have demonstrated that deltamethrin has the ability to increase the activity of PKC and the production of inositol tri- and tetraphosphate in rat brain synaptosomes.

EXPERIMENTAL PROCEDURES

Materials. $H_3{}^{32}PO_4$ (1 mCi, 200 mCi/mmol), [γ-^{32}P]ATP (250 μCi; 5000 Ci/mmol) and *myo*[2-^{3}H]-inositol (1 mCi/mL; 18.2 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). Deltamethrin [(*S*)-α-cyano-3-phenoxybenzyl *cis*-(1*R*, 3*R*)-2,2-dimethyl-3-(2,2-dibromovinyl) cyclopropane carboxylate] was a gift from Roussel Uclaf. All other biochemicals were purchased from the Sigma Chemical Co. (St. Louis, MO) and were of the highest purities available.

Preparation of the synaptosomes (P2 fraction). Intact synaptosomes were prepared from whole brain tissue of young adult rats (Sprague–Dawley) and washed in modified-Krebs buffer as previously described by Robinson and Dunkley [15]. They were prepared fresh for each *in situ* test and were used within 6 hr of killing the animals.

Phosphorylation studies. Samples of the intact P2 fraction (500 μg protein) were prelabeled with 0.2 mCi/mL $^{32}P_i$ in 200 μL of Krebs-bicarbonate buffer, pH 7.4, at 37°, bubbled with 5% CO_2:95% O_2 [16]. Deltamethrin (usually 0.1 μM final concentration) was added at 35 min and the prelabeling was continued until a total of 45 min elapsed.

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† Abbreviations: PKC, protein kinase C; IP_3 or Ins(1,4,5)P_3, inositol 1,4,5-triphosphate; IP_4 or Ins(1,3,4,5)P_4, inositol 1,3,4,5-tetraphosphate; PI, phosphoinositol; TPA, 12-*O*-tetradecanoylphorbol-13 acetate; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PMB, polymyxin B; and AF, auranofin.

Depolarization was initiated by adding an aliquot (40 μL) of depolarizing buffer [7, 15] to raise the K^+ concentration to 62 mM. After various time intervals depolarization was terminated by the addition of a sodium dodecyl sulfate (SDS) containing "stop reagent" [7, 16], followed by immediate transfer to ice. In a parallel experiment, 12-*O*-tetradecanoylphorbol-13 acetate (TPA) was added 5 min prior to the depolarization (i.e. 5 min after the addition of deltamethrin) and the synaptosomes were depolarized for different time intervals.

Polyacrylamide gel electrophoresis (PAGE). The techniques utilized to obtain one-dimensional SDS–PAGE and autoradiography of ^{32}P-labeled proteins were as described previously by Enan and Matsumura [7]. Two-dimensional SDS-PAGE for ^{32}P-labeled synaptosomal proteins was carried out by the method of O'Farrell [17] with the following modifications: The synaptosomal preparation was prelabeled with 0.5 mCi of $^{32}P_i$ and depolarized for 60 sec with the depolarization buffer to induce a "high K^+" (62 mM) KCl condition as described above. The reaction was stopped by the addition of an equal volume of the "sample buffer" (9.5 M urea, 2% NP-40, 1.6% ampholyte, pH 5/7, 0.4% ampholyte, pH 3/10, and 5% 2-mercaptoethanol). This was then mixed and immediately placed on ice. The first gel tubes were run at 400 V for 13 hr and thereafter the voltage was increased to 800 V for 1 hr. The gel was removed from the tube and placed along the top edge of a 10% SDS–polyacrylamide slab gel and electrophoresed for the second dimension [7].

PKC preparation, purification, and assay. Synaptic plasma membrane from albino male rat brain was prepared [18] and solubilized, and PKC was partially purified from this preparation using DEAE-cellulose columns [14]. A 40-μg protein equivalent of the purified enzyme in 20 μL of 0.3 M NaCl was incubated with the test concentrations of deltamethrin for 10 min at 25°, followed by the addition of 50 μL of the assay buffer [20 mM Tris–HCl pH 7.5, 2 mM ethyleneglycolbis (aminoethylether) tetra - acetate (EGTA), 5 mM $MgCl_2$, containing usually 2 mM dithiothreitol (DTT), 5 μM ATP and 1.83 mM $CaCl_2$], 2 μg dioctanylglycerol, 40 μg of calf thymus histone type III S and 5 μg phophatidylserine. The ^{32}P labeling was initiated by the addition of 0.5 μCi of [γ-^{32}P]ATP and incubation for 10 min at 25°. An aliquot (20 μL) was then spotted onto a piece of phosphocellulose paper [14]. The papers were washed three times with 80 mM phosphoric acid and the remaining radioactivity on the paper was counted by liquid scintillation spectrometry. The enzyme activity obtained in the absence of phosphatidylserine (but in the presence of all other cofactors) was subtracted from all of the experimental data to obtain the net PKC activity in terms of incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP into calf thymus histone (type III S). At the same time, the effects of TPA, neomycin, polymyxin B and auranofin were studied on partially purified PKC. TPA (10^{-6} M) was added 5 min after the addition of deltamethrin, while the others (neomycin 200 μM, polymyxin B 10 μg/mL and auranofin 40 μM) were added 5 min prior to the addition of deltamethrin. For comparative potency studies, rat brain synaptosomes were incubated *in vitro* with a 0.1 μM concentration of two isomers of deltamethrin (1*R* and 1*S*), active isomers of fenvalerate (Aα) and cypermethrin for 10 min at 37°. The cytosolic and particulate PKC were separated and partially purified with DEAE-cellulose columns, and the enzyme activity in each fraction was measured as described above.

Inositol phosphate determination. Rat brain slices (350 μm) from the whole brain were used to measure the effect of 0.1 μM deltamethrin (1*R*) on the inositol phosphate levels. Brain slices were prepared [19] and 3 μCi of *myo*[2-^{3}H]inositol was added to 50 μL of gravity packed rat brain slices and incubated for 90 min at 37°. The prelabeled tissues were washed thoroughly for 60 min at 37° with Krebs–Ringer buffer, pH 7.5, containing a 10 mM concentration of nonlabeled *myo*-inositol. The washed slices were gassed (95% O_2, 5% CO_2), covered and incubated at 37° for 10 min prior to the addition of 0.1 μM deltamethrin (or 1 mM carbachol as a positive control). After incubation with deltamethrin or carbachol for 10 min, the reactions were terminated by the addition of 1 mL of chloroform/methanol (1:2) [18]. For another set of tests, LiCl (10 mM) was added and the same experiment was prepared. The liquid phase was collected and the brain slices were then homogenized in 200 μL of 0.5 N HCl. The two fractions were then combined. To this mixture, 200 μL each of distilled water and chloroform were added, mixed well, and centrifuged at 3000 *g* for 15 min. One milliliter of the aqueous phase was mixed with 3.3 mL of distilled water and transferred to columns, each containing 1 mL of Dowex-1 (X8, formate form, 200–400 mesh). The labeled metabolic products were eluted by the stepwise addition of solutions containing increasing levels of formate [18–21]. At the same time, different concentrations of deltamethrin (10^{-10}–10^{-7} M) were incubated with rat brain slices and the total inositol polyphosphates were measured as described above, except that the total products were eluted one time using a solution containing 1.0 M formate.

RESULTS

Protein phosphorylation. To confirm the previously observed stimulatory property of deltamethrin on synaptic protein phosphorylation [7], we isolated rat brain synaptosomes, labeled them with $^{32}P_i$, and depolarized them by increasing the external K^+ concentration in the presence and absence of 0.1 μM deltamethrin (Table 1). The resulting scanning of the SDS–PAGE gel using a computerized scanner (Ambis) showed that the insecticide caused a significant increase in the level of protein phosphorylation. This effect was observable even 5 min after depolarization, when the level of depolarization-induced phosphorylation on total proteins had declined to the predepolarization level in control synaptosomes. It was noted at the same time that this deltamethrin-induced increase in phosphorylation was particularly significant for a few specific proteins, i.e. phosphoproteins of 87, 48, and 38 kDa. The characteristics of these phosphoproteins were analyzed next using two-dimensional SDS–PAGE (Fig. 1). Deltamethrin was found to cause a

Table 1. Effect of deltamethrin (0.1 μM) on depolarization-dependent protein phosphorylation in rat brain synaptosomes

Depolarization time (sec)	Phosphorylation (% of control)					
	Control			Deltamethrin		
	38 kDa	48 kDa	87 kDa	38 kDa	48 kDa	87 kDa
0	100 ± 9	100 ± 5	100 ± 11	100 ± 9	100 ± 7	100 ± 9
15	97 ± 3	118 ± 10	107 ± 8	122 ± 8*	125 ± 5*	125 ± 12*
300	95 ± 6	112 ± 8	110 ± 5	125 ± 7*	134 ± 12†	132 ± 7†

The phosphorylation value at 0 min depolarization was adopted as 100% for each protein band. The absolute values of protein phosphorylation at 0 min depolarization for proteins 38, 48 and 87 kDa, respectively, were: control: 3900 ± 120, 2750 ± 240 and 3570 ± 310 dpm; deltamethrin: 4000 ± 310, 2800 ± 250 and 3700 ± 400 dpm. Data have been derived through the scanning of a radioautogram of a one-dimensional SDS–polyacrylamide gel electrophoretogram using a computerized image scanner (Ambis). This experiment was repeated three times and the data shown are means ± SD.

*† Significantly different from the control value: *$P \leq 0.05$ and †$P \leq 0.01$ (Cochran's t-test).

significant increase in phosphorylation of two very acidic proteins with molecular weights of 87 and 48 kDa, and one of a basic 38 kDa protein.

Effects of deltamethrin and other chemicals on PKC activity. To confirm that the protein phosphorylation activities found in this partially purified preparation included that of PKC, we used some specific inhibitors and a promoter separately in the incubated PKC assay mixture. A significant stimulation of enzyme activity was found in the presence of TPA, while the phosphatidylserine-requiring kinase activity was inhibited significantly in the presence of neomycin, polymyxin B (PMB) and auranofin (AF) as expected (Fig. 2). While deltamethrin stimulated protein phosphorylation in all combinations, the degree of its stimulation was smaller in the presence of these specific inhibitors.

To assess the direct effect of deltamethrin on PKC, partially purified rat brain PKC was preincubated with various concentrations of deltamethrin. Using incorporation of [γ-^{32}P]ATP into histone as a criterion [14], it was clearly demonstrated that PKC activity was stimulated by very low concentrations of deltamethrin (Fig. 3). To determine whether this action of deltamethrin also occurs in intact synaptosomes on native protein substrates as well, synaptosomes were prelabeled with [^{32}P]-phosphoric acid, treated with TPA alone or TPA plus deltamethrin, and depolarized, the changes in the level of ^{32}P-phosphorylation on native proteins were monitored using the SDS–PAGE method. The results clearly showed that TPA increased the depolarization-dependent protein phosphorylation after 5 sec of depolarization, and this increase was depolarization time dependent. Moreover, deltamethrin was found to potentiate the stimulatory effect of TPA when the former was added just 5 min before the latter (Fig. 4).

Effects of deltamethrin and carbachol on phosphoinositide turnover and the translocation of PKC. It is known that activation of PKC is accompanied by increased formation of diacylglycerol and inositol 1,4,5-triphosphate (IP_3) [22, 23], with a subsequent increase in the intracellular concentration of Ca^{2+}. Employing the method described by Kolulainen and Bondy [24], we assessed free intracellular Ca^{2+} of intact synaptosomes in Krebs-bicarbonate buffer and found that its levels were 120 ± 15 and 77 ± 13 nM for the deltamethrin-treated and control (treated with 1 μL of ethanol only) preparations, respectively. After depolarization with K^+ (final concentration 33.5 mM) for 30 sec, the corresponding values rose to 167 ± 9.6 and 100 ± 11 nM, respectively.

The action of deltamethrin on phosphoinositide metabolism was studied next, this time using rat brain slices. Deltamethrin produced a clear increase in intracellular phosphoinositide levels. Moreover, the effect of deltamethrin was even more potent than that of carbachol and more pronounced in the presence of 10 mM LiCl (Table 2). This deltamethrin-induced increase of total polyphosphates was concentration dependent (Fig. 5). At the highest concentration tested, deltamethrin induced an approximately 40% increase in total inositol polyphosphate levels.

As expected, the addition of 200 μM neomycin resulted in a reduction in the level of the phosphoinositol (PI) metabolites in both control and carbachol-treated preparations. Nonetheless, under such a condition deltamethrin still produced an elevation in IP_3 levels beyond that of the matching brain slices treated with neomycin alone (Table 2).

Finally, we examined the translocation of PKC from a cytosolic location to the membrane fraction under the influence of a 0.1 μM concentration of several synthetic pyrethroids and DDT. The data (Table 3) revealed that all active forms of pyrethroids and DDT promoted translocation of PKC to the membrane fraction of the synaptosomal preparations.

DISCUSSION

In the current work, we have obtained several pieces of evidence indicating that pyrethroids stimulate the phosphoinositide/PKC pathway. In the synaptosomal preparations we noted that

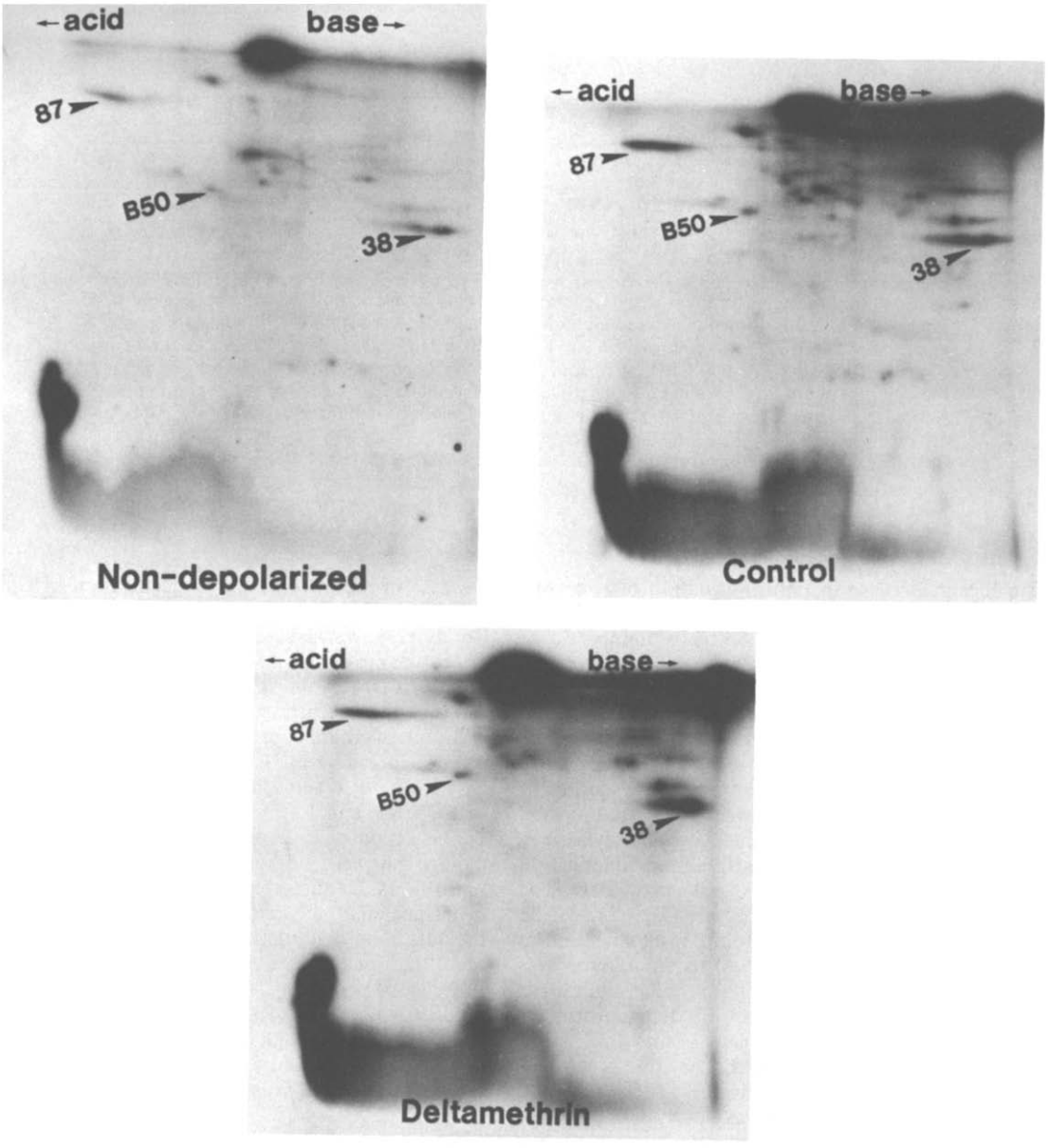

Fig. 1. Two-dimensional SDS–PAGE of ^{32}P-labeled synaptosomal phosphoproteins. Assay conditions and the synaptosomal preparation used were as described in Experimental Procedures. The synaptosomes were labeled with 0.5 mCi of $H_3{}^{32}PO_4$ and the depolarization period was 60 sec. The reaction was stopped by adding an equal volume of the "sample buffer," and two-dimensional PAGE was developed. Key: (left panel) non-depolarized, (right panel) control, and (bottom panel) deltamethrin-pretreated synaptosomes; in the bottom and right-hand panels, synaptosomal preparations were depolarized for 60 sec, using 62 mM KCl in Krebs–Ringer bicarbonate buffer, pH 7.4.

among [^{32}P]phosphoproteins, deltamethrin caused a particularly significant increase in the phosphorylation of two very acidic proteins of 48 kDa (= B50 protein) and 87 kDa. These two acidic proteins have been reported previously to be preferred substrates for PKC [10, 16, 26] in rat brain synaptosomes. Indeed, when the experiment was repeated using TPA, a well established promoter of PKC, qualitatively the same results were obtained (Fig. 4).

A question must be raised here as to whether such an action of deltamethrin is due to its direct action on PKC or on other systems which modulate the intracellular environment of the intact synaptosome. For this reason, we partially purified rat brain PKC with a DEAE-cellulose column and assessed the direct effect of deltamethrin on this enzyme with various concentrations. The results (Fig. 3) clearly indicate that very low concentrations of deltamethrin could directly stimulate PKC.

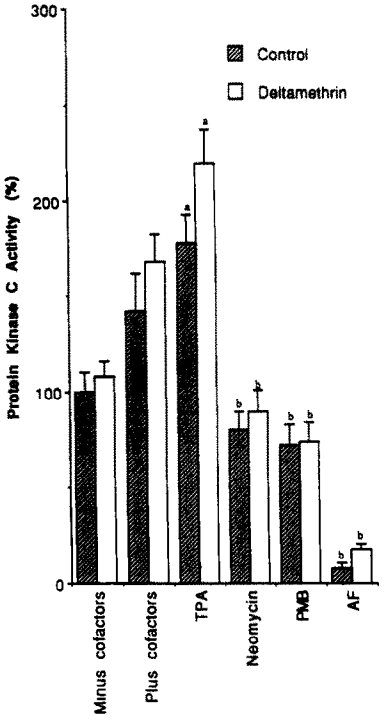

Fig. 2. Assay of PKC activity. Synaptic plasma membrane was prepared and solubilized, and PKC was purified from this preparation using DEAE-cellulose columns. The partially purified enzyme was directly preincubated with the test chemicals with and without 10^{-7} M deltamethrin for 10 min at 25°. After addition of calf thymus histone type III S and PKC cofactors, the reaction was initiated by the addition of 0.5 μCi of [γ-^{32}P]ATP and terminated by spotting on phosphocellulose paper for determination of phosphorylation activity. Vertical lines at individual data points indicate the standard deviation of three experiments. The absolute value of phosphorylation in the absence of cofactors was 6.9 ± 0.1 mol ^{32}P/10 μg histone/10 min. The values shown are a percentage of this value. Key: (a) and (b) significantly different from control values (plus cofactors) [$P \leq 0.05$ or $P \leq 0.01$, respectively, Cochran's *t*-test].

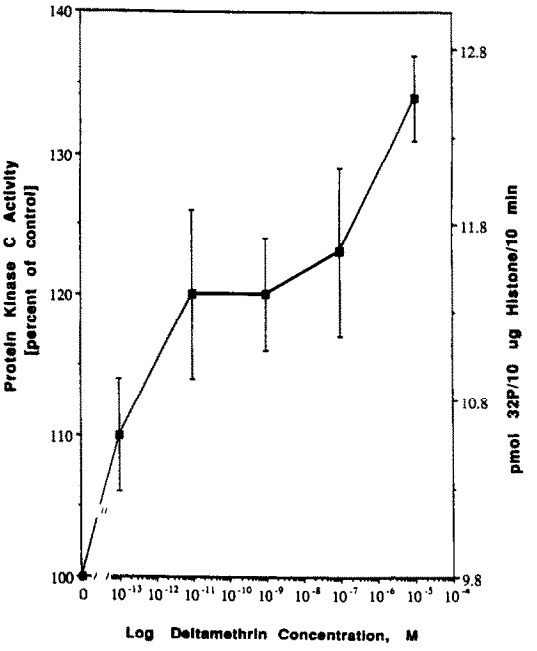

Fig. 3. Stimulation of isolated synaptic PKC by deltamethrin. The synaptic plasma membrane from male rat brain was prepared and solubilized, and PKC was purified from this preparation using DEAE-cellulose columns. The partially purified enzyme was directly preincubated with the test concentrations of deltamethrin for 10 min at 25°. After addition of calf thymus histone type III S and PKC cofactors, the reaction was initiated by the addition of 0.5 μCi of [γ-^{32}P]ATP and terminated by spotting on phosphocellulose paper for determination of phosphorylation activities. Vertical lines at individual data points indicate standard deviations of four independent experiments, each run in triplicate.

The possibility that deltamethrin also affects other components of phosphatidylinositol metabolism was studied next using rat brain slice preparations. The results showed that deltamethrin increased intracellular levels of phosphoinositides, and that it was more potent in this regard than carbachol (Table 2), which was used as a positive control [19]. The EC_{50} of deltamethrin was approximately 0.1 μM. While the effects of carbachol could be totally suppressed by 0.1 mM atropine (data not shown), those of deltamethrin were only partially antagonized by this treatment. When the experiment was repeated with isolated synaptosomes in place of brain slices, carbachol was not found to produce a significant effect on the rate of phosphoinositide production, whereas deltamethrin treatment still clearly increased phosphoinositide level (data not shown). These two sets of data suggest that it is unlikely that the membrane bound receptor portion of the phosphoinositide pathway is a primary site of action of deltamethrin. Moreover, the effect of deltamethrin was even more pronounced in the presence of Li^+, which was added at a concentration sufficient to inhibit the action of most cellular phosphatases [18, 19], indicating that the action of deltamethrin is not on the process of degradation of these phosphoinositides.

The administration of 200 μM neomycin to partially block the formation of IP_3 and inositol 1,3,4,5-tetraphosphates (IP_4) resulted in a reduction in the level of these PI metabolites in both control and carbachol-treated preparations as expected. However, under such a condition deltamethrin still elevated the level of IP_3 beyond that of the neomycin-untreated control. The logical conclusion is that the direct action site of deltamethrin is not on the PI metabolic cycle (e.g. phospholipase C, PI monophosphokinase); rather its effect is likely to be on PKC, an IP_3-independent Ca^{2+} triggering site (e.g. ryanodine receptor, endoplasmic reticulum stores and Ca^{2+} channel) and/or phosphoprotein phosphatase [27]. Since deltamethrin itself does not

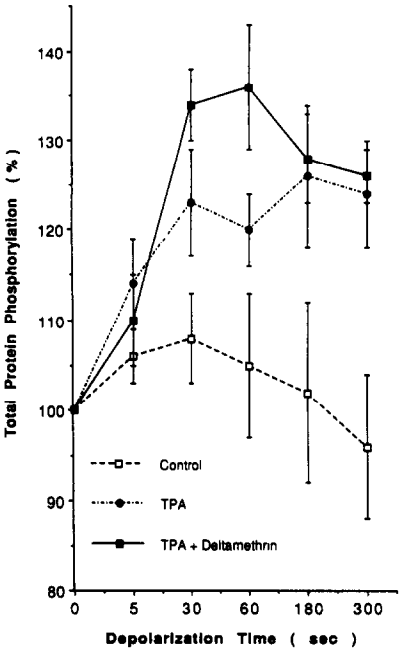

Fig. 4. Effects of TPA and deltamethrin on protein phosphorylation activities in intact synaptosomes. Assay conditions and the synaptosomal preparation used were as described in the legend of Fig. 1, except that the synaptosomes were depolarized for different time intervals (0–300 sec). TPA (10^{-6} M) was added 5 min prior to depolarization, while deltamethrin was added 10 min prior to depolarization. The results were analyzed using one-dimensional SDS–PAGE. Total phosphorylation on native synaptic proteins was used as the criterion for protein kinase activities. Data are expressed as the means ± SD of three independent experiments. Each experiment was run in triplicate. The absolute value of protein phosphorylation at 0 min depolarization was 10,950 ± 420.

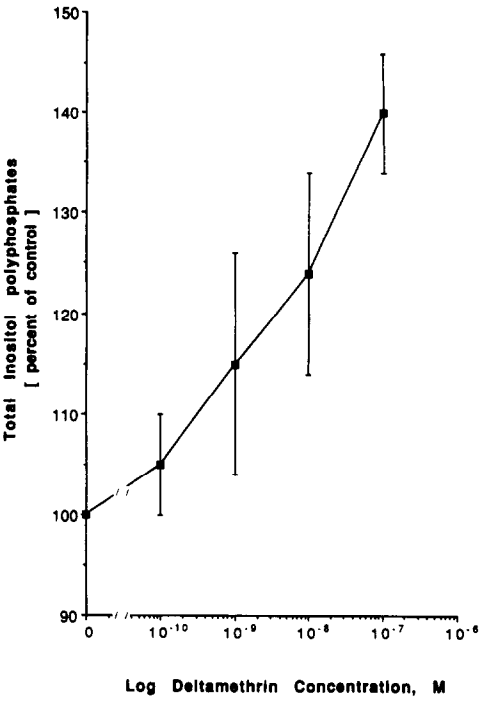

Fig. 5. Effect of deltamethrin on metabolic formation of total inositol polyphosphates ($IP_2 + IP_3 + IP_4$) in rat brain slices. Rat brain slices were used to measure the effect of 10^{-7} M deltamethrin on the inositol levels using *myo*[2-^{3}H]-inositol. The labeled metabolic products of total inositol polyphosphates were eluted by the addition of solution containing 1.0 M formate. Data are expressed as the means ± SD of four independent experiments. Each experiment was run in duplicate. The absolute value of total inositol polyphosphate in the control (without deltamethrin) was 37,920 ± 2,340 dpm.

Table 2. Effects of deltamethrin (0.1 μM) and carbachol (1 mM) on the levels of water-soluble inositol metabolites in rat brain slices

Treatment	Inositol	Glycerophosphoinositol	Ins 1P*	Ins (1,4)P_2*	Ins (1,4,5)P_3*	Ins (1,3,4,5)P_4*
NaCl (123 mM)						
Control	30,520 ± 1,220	1,810 ± 110	2,250 ± 90	1,180 ± 50	1,000 ± 100	250 ± 20
Carbachol	39,320 ± 2,100	3,980 ± 250†	3,210 ± 100‡	1,390 ± 40†	1,060 ± 100	250 ± 30
Deltamethrin	39,280 ± 980	2,780 ± 80‡	3,220 ± 101‡	1,600 ± 40†	1,300 ± 80†	250 ± 50
LiCl (10 mM)						
Control	55,320 ± 140	1,740 ± 40	3,350 ± 70	3,090 ± 180	2,740 ± 50	390 ± 20
Carbachol	51,510 ± 930	2,220 ± 40‡	4,190 ± 50†	1,880 ± 50†	1,940 ± 40†	340 ± 20
Deltamethrin	86,960 ± 580†	3,560 ± 160†	5,710 ± 40†	3,670 ± 100	3,560 ± 30†	500 ± 10‡
Neomycin (200 μM)						
Control	21,320 ± 840	890 ± 70	1,440 ± 30	820 ± 20	1,010 ± 100	240 ± 40
Carbachol	14,900 ± 680†	850 ± 60	1,020 ± 10†	920 ± 50	1,420 ± 40†	240 ± 20
Deltamethrin	22,810 ± 110	1,440 ± 20‡	1,610 ± 40†	1,210 ± 40†	1,510 ± 30†	240 ± 20

Values are expressed as ^{3}H-dpm (means ± SD) from five independent experiments.
* Abbreviations used are as described by Irvine *et al.* [25].
† Significantly different from control value ($P \leq 0.01$, Cochran's *t*-test).
‡ Significantly different from control value ($P \leq 0.05$, Cochran's *t*-test).

Table 3. Effects of selected insecticides (all tested at 0.1 μM) on PKC of rat brain synaptosomes

Compound (isomer)	Cytosol fraction	Plasma membrane fraction	% in Membrane fraction
Control	3.6 ± 0.8	1.2 ± 0.1	25
Deltamethrin (1*R*)	2.1 ± 0.1	3.8 ± 0.5	55*
Deltamethrin (1*S*)†	3.0 ± 0.7	1.8 ± 0.8	38
Fenvalerate (Aα)	1.9 ± 0.9	5.0 ± 0.2	72*
Cypermethrin	1.6 ± 1.1	2.7 ± 0.6	63*
Allethrin	2.0 ± 0.4	2.8 ± 0.1	58*
DDT	2.0 ± 0.6	5.0 ± 1.6	71

Data are expressed as ^{32}P incorporated from [γ-^{32}P]ATP in terms of pmole ^{32}P/10 μg histone/10 min).

Results are means ± SD from four independent tests.

* Significantly different from control values ($P \leq 0.001$, Cochran's *t*-test).

† The inactive stereoisomer of deltamethrin.

cause an increase in transmitter release in the case of isolated rat brain synaptosomes in the absence of depolarization [5, 6], and since its effects are manifested even when external Ca^{2+} is replaced with Ba^{2+} [7], the Ca^{2+} channels may be eliminated from the consideration. One system which is potentially important in this regard is Ca^{2+}-Mg^{2+}-ATPase. However, its sensitivity toward type II pyrethroids is not as high [28] ($EC_{50} = 3 \times 10^{-8}$ M) as that of PKC.

Finally, we have addressed the question of the persistence of the effect of deltamethrin's on the neurotransmitter release processes. Recent studies have shown that in many cases PKC could move from a cytosolic locale to the plasma membrane with persistent stimulation (e.g. long-term potentiation treatments) which in turn induces long-term facilitation of synaptic transmission [11, 14, 26]. The survey of the relative levels of PKC in the plasma membrane versus the cytosolic sites of synaptosomal preparations (Table 3) revealed that all active forms of type II pyrethroids promote translocation of PKC to the plasma membrane.

There are a number of recent reports addressing the functional meaning of PKC in synaptic processes [8, 29]. In the presynaptic terminal, the phosphoinositide/PKC pathway apparently acts as a signal amplifying mechanism for depolarization- or receptor-induced neurotransmitter releasing processes [10, 30]. For instance, phorbol esters [30, 31] have been shown to cause amplification of the quantity and the duration of quantal neurotransmitter release, which becomes observable only when the synapse is depolarized. Such a conclusion is a reasonable one in view of the usual presence of a very high titer of PKC in both pre- and postsynaptic terminals and the apparent needs for signal amplifying mechanisms in neural processes for memory formation and retention [14].

The most important aspect of the effects of pyrethroids described here is that the compounds of this group do not induce depolarization by themselves as in the case of phorbol esters. Rather, their actions are seen only when the plasma membrane is depolarized by electric stimuli or by chemical treatments (such as veratridine) that induce depolarization (i.e. use-dependent action) [6, 15]. Such an action pattern of type II pyrethroids indicates that they may be amplifying incoming signals, especially in neurons rich in PKC [32] and/or components of the phosphoinositol pathway [33]. Particularly interesting is the cerebellar region where type II pyrethroids are known to cause a sharp rise in cGMP levels [34], probably because of an increase in glutamate type transmitter release by parallel fibers adjacent to Purkinje cells [35]. Another region rich in PKC is the hippocampus, which plays an important role in "long-term potentiation." This process is also known to involve activation and translocation of PKC to plasma membrane sites [14]. While we have not specifically shown in the current work that PKC in these brain regions is affected by the pesticides, earlier behavioral work [2–4, 8, 36] does suggest that the cerebellum and hippocampus may be involved in the action of these pesticides.

Certainly much more work is needed in this area. However, in view of the importance of this subject area, our current findings provide a fresh look at the unusual neurotoxicological properties of these pesticides.

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