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INTERACTION OF DDT AND PYRETHROIDS WITH CALMODULIN AND ITS SIGNIFICANCE IN THE EXPRESSION OF ENZYME ACTIVITIES OF PHOSPHODIESTERASE

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Abstract—To understand the significance of the inhibitory action of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) and pyrethroid insecticides on calmodulin, a universal Ca^{2+} binding protein, a bovine heart phosphodiesterase–calmodulin system was studied. It was found that, at concentrations of less than 10^{-5} M, the inhibitory action of DDT of the phosphodiesterase was due entirely to its action on calmodulin alone. Cypermethrin was less potent than DDT, but it also affected only calmodulin. Permethrin was the most potent inhibitor affecting calmodulin and, to a lesser extent, phosphodiesterase. The inhibitory action of these insecticides on calmodulin raises a possibility that many unsuspected Ca^{2+}-related systems are being affected by these insecticidal chemicals, as calmodulin is known to play vital roles in many biological reactions dependent upon Ca^{2+}. These include modulation of phosphodiesterase, neurotransmitter release, adenylate cyclase, Ca^{2+}-dependent protein kinase, myosine light chain kinase and various membrane phosphorylation systems.

It has been clearly established that various chlorinated and pyrethroid pesticides affect systems which are engaged in calcium transport (e.g. Ca^{2+}-Mg^{2+}-ATPase, Na^+/Ca^{2+} exchange and mitochondrial Mg^{2+}-ATPase) or those which are controlled by changes in Ca^{2+} concentration: e.g. sodium channel, ACh-receptor, and cAMP-dependent protein kinase (see review by Matsumura [1], and also Matsumura and Clark [2]). Since the activities of many of these Ca^{2+}-influenced or -dependent systems are now known to be controlled by calmodulin, a universal Ca^{2+} binding protein, a natural question may be raised as to its role in the overall inhibitory actions of these insecticides.

However, unpurified enzyme preparations such as Ca^{2+}-Mg^{2+}-ATPase in the nerve homogenates usually contain unspecified amounts of calmodulin. In some preparations, elimination of calmodulin is not easy, and even repeated washings with chelating agents such as ethyleneglycol *bis*(amino-ethyl-ether)tetra-acetate (EGTA) do not completely provide calmodulin-free preparations [3]. Thus, even the cases where a clear-cut inhibition of the system is established in the absence of added exogenous calmodulin, one cannot conclude whether the enzyme itself is inhibited or the effect is due to inhibition of associated calmodulin.

The relationship of the enzyme phosphodiesterase to calmodulin was originally established by Cheung and later by many other scientists (see Ref. 4). Its high sensitivity to calmodulin and the availability of highly purified forms make this enzyme ideal for this type of investigation. Recently, Hagman [5] has suggested that 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) inhibits calmodulin. He based his conclusion on the ability of excess calmodulin to reverse the inhibition of phosphodiesterase by DDT. However, such a phenomenon can be caused by a variety of factors (see discussion below). In view of the importance of the subject matter, we decided to examine the details of the interaction of DDT with the phosphodiesterase–calmodulin complex.

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EXPERIMENTAL

The bovine heart phosphodiesterase used for this study was purchased from the Sigma Chemical Co. (Product No. P-0520, Lot No. 23F-9570). It is termed "activator deficient" and is provided as a lyphophilized powder. Usually 2 units of phosphodiesterase were dissolved per 1 ml of 20 mM imidazol buffer, containing 100 μM $CaCl_2$ and 5 mM of $MgCl_2$ at pH 7.4. The method of the enzyme assay was that of Wolff *et al.* [6] as used by Siegel and Haug [7], with [^{3}H]guanosine, 3,5-cyclic phosphate (ammonium salt), purchased from New England Nuclear, Inc. (12.1 Ci/mmole), as a substrate. To an aliquot of 214 μl of the above imidazol buffer, 10 μl of cGMP (final concn = 25 μM) and 75 μl of calmodulin (final concn = 344 nM in the standard condition) in the same buffer were added. The insecticide was added with 1 μl of ethanol, control receiving 1 μl of ethanol. The system was preincubated for 10 min at 0°. The reaction was started by the addition of 1 μl of the enzyme, and the system was incubated at 37° for 3 min. The reaction was terminated by boiling for 2 min, and cGMP remaining was treated with 5′-nucleotidase and then with the ion-exchange resin as described by Siegel and Haug [7].

The levels of DDT bound to calmodulin were assayed as follows: 750 μl calmodulin (final concn 344 nM) was added to 2240 μl of the same buffer, and preincubated for 10 min at 0°. DDT was added with 10 μl ethanol (DDT final concn 10^{-5} M), and the system was incubated for 3 min at 37°. The reaction was terminated with 2 ml of the buffer containing 3% charcoal and 0.03% dextran (w/v), the system was shaken vigorously with a vortex mixer for 1 min and then was centrifuged at 3000 *g* for 10 min. The supernatant fraction was collected and extracted three times, each time with 1 ml of ether. The tube containing the supernatant fraction was rinsed with *n*-hexane. The solvents were combined and evaporated, and the residue was redissolved in 10 ml of *n*-hexane and analyzed for DDT through gas-liquid chromatographic analysis (GLC). The amount of DDT bound to calmodulin was determined by subtracting the value obtained in the absence of calmodulin from the value obtained in the presence of calmodulin.

In a parallel experiment, the reaction was stopped by boiling and the product was eluted through a 1.5 × 30 cm Sephadex G.75 column using the same buffer. The eluted fractions corresponding to the position of calmodulin were extracted, and DDT levels were determined by GLC as above. Each assay was repeated three times.

Calmodulin was obtained using the isolated procedure of Caldwell and Haug [8] from the acetone powder preparation of the bovine brain from the Sigma Chemical Co.

The following chemicals were obtained from respective manufacturers: DDT (Aldrich Chemical Co.) (purity 99%); cypermethrin (FMC) (technical grade, (*R,S*)-α-cyano-3-phenoxybenzyl(±)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate); deltamethrin (Roussel Uclaf) (99.6%, (*R,S*)-α-cyano-3-phenoxybenzyl(±)*cis,trans*-3-2,2-dibromovinyl)-2,2-dimethylcyclopropane carboxylate); allethrin (Fairfield American Corp.) (90%, *S*-α-allethrolone(+)-*trans*-3-(2,2-dimethylvinyl)-2,2-dimethylcyclopropane carboxylate); permethrin (Penick Corp.) (91%, 3-(phenoxyphenyl) methyl(±)-*cis,trans*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane carboxylate).

RESULTS

The effects of calmodulin and 10^{-5} M DDT were first studied on the phosphodiesterase activity in the presence and the absence of Mg^{2+} (Table 1). In the presence of Mg^{2+}, the effect of calmodulin was more pronounced on phosphodiesterase activity as previously shown by Brostrom and Wolff [9]. DDT at this concentration was inhibitory whether there was Mg^{2+} or not, so long as exogenously added calmodulin was present. Since high degrees of calmodulin stimulation were desirable for the current investigation, we elected to use Mg^{2+} in all subsequent tests.

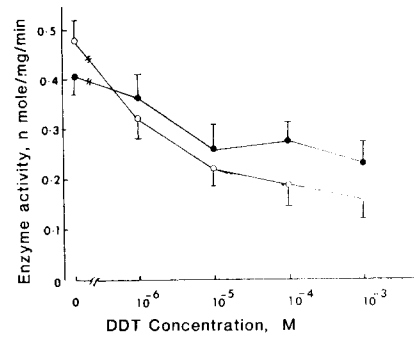

Fig. 1. Inhibition of calmodulin-stimulated phosphodiesterase activity by DDT. In Method A (i.e. the regular incubation condition), 10^{-5} M DDT was preincubated with 25 μM cGMP, 344 nM calmodulin and 100 μM $CaCl_2$ in 20 mM imidazole, pH 7.4 at 0° for 10 min. The enzyme was added to initiate the reaction at 37° for 3 min (○). In method B (●), the enzyme was added first and preincubated for 10 min with DDT in the same buffer as above. The reaction was started by the addition of [^{3}H]cGMP 25 μM and 344 nM calmodulin at 37° for 3 min. The vertical lines indicate the standard error for each point. Average of three experiments.

To study whether DDT is preferentially inhibiting calmodulin or phosphodiesterase, the following experiment was devised. In the regular assay, DDT was preincubated with calmodulin for 10 min at 0° and the enzyme was added to initiate the reaction (method A). However, in this experimental a parallel assay was conducted by using method B where the enzyme was added first to the buffer, preincubated with DDT for 10 min at 0° and the reaction started by the addition of [^{3}H]cGMP plus calmodulin.

The results shown in Fig. 1 clearly indicate that, at all DDT concentrations, the phosphodiesterase activity was better inhibited with the regular approach (method A), where calmodulin was first allowed to come in contact with DDT.

Table 1. Effects of calmodulin, Mg^{2+} and DDT on the phosphodiesterase activity*

Treatment	Enzyme activity (nmoles/mg/min)	
	Without addition of Mg^{2+}	Presence of Mg^{2+}
None (= control)	0.115 ± 0.031	0.094 ± 0.039
DDT	0.126 ± 0.028	0.073 ± 0.092
Calmodulin	0.230 ± 0.025	0.255 ± 0.120
DDT + calmodulin	0.157 ± 0.025	0.193 ± 0.210

* Concentrations were: calmodulin, 344 nM; DDT, 10^{-5} M; and Mg^{2+}, 5 mM.
Data are expressed as mean ± S.D. of three to six determinations.

Table 2. Inhibitory effects of DDT and chlorpromazine on the calmodulin-stimulated phosphodiesterase activity*

Treatment	Enzyme activity† (nmoles/mg/min) Without calmodulin	With calmodulin	Calmodulin stimulation	% Inhibition
None	0.217 ± 0.008	0.880 ± 0.088	0.663	
DDT	0.183 ± 0.009	0.573 ± 0.005	0.390	41.2
Chlorpromazine	0.140 ± 0.002	0.318 ± 0.004	0.178	73.2
DDT + chlorpromazine	0.108 ± 0.001	0.245 ± 0.022	0.137	79.3

* Concentrations were: DDT, 10^{-5} M; chlorpromazine, 10^{-4} M; and calmodulin, 344 nM.
† Data are expressed as mean ± S.D. of three to six determinations.

One of the agents that has been acknowledged to specifically inhibit calmodulin is chlorpromazine [10]. Therefore, the inhibitory action of chlorpromazine was first tested under the standard assay method. The I_{50} of chlorpromazine was determined to be around 10^{-5} M. At 10^{-4} M approximately 70% of the total enzyme activity was suppressed (data not shown). DDT (10^{-5} M) and chlorpromazine (10^{-4} M) were added separately and together (Table 2). DDT at this dose inhibited approximately 40% of the calmodulin-stimulated portion of the activity, whereas in the presence of chlorpromazine, DDT (10^{-5} M) inhibited only 23% of the calmodulin stimulation. These data indicate that these two agents have the capability to affect calmodulin, but these data alone, however, are not sufficient to establish that DDT affects only calmodulin. Therefore, another set of experiments was devised where the amount of calmodulin in relation to phosphodiesterase was varied (Fig. 2). When the reciprocals of calmodulin concentration and the enzyme activity (Lineweaver–Burk plot [11]) were plotted, the lines were found to intersect at the ordinate. This means that the effect of DDT could be completely overcome when a large amount of calmodulin was added. Such a phenomenon could be explained, if (a) DDT inhibits only calmodulin, (b) the inhibition of DDT on phosphodiesterase is completely reversible, and calmodulin either directly competes at the same site on the enzyme or at a close or tightly coupled site to be allosterically affected by calmodulin–Ca^{2+} binding, or (c) the action of DDT is antagonized by the increase in the effective dose of calcium as a result of calmodulin's action.

In the classic reversible inhibition cases, the level of the substrate is known to affect the degree of inhibition. When the cGMP concentration was varied at a fixed calmodulin concentration, an unusual relationship was obtained (Fig. 3). Here in the control tests the substrate–activity relationship followed a typical Lineweaver–Burk function, whereas that for DDT treated did not. The most plausible explanation of the phenomenon is that at low substrate concentrations the role of calmodulin is not as critical, whereas at high substrate concentrations all available calmodulin molecules are mobilized and used maximally. Thus, DDT inhibition of available calmodulin becomes much more critical at high substrate concentrations. Whether this conclusion is correct or not, the above relationship at least rules out the possibility that DDT inter-

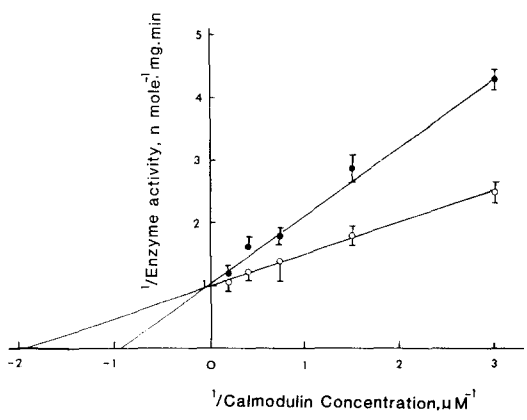

Fig. 2. Double-reciprocal plot of the calmodulin-stimulated phosphodiesterase activity vs calmodulin concentrations in the absence (○) and presence (●) of DDT (10^{-5} M). See Fig. 1 for other conditions (method A).

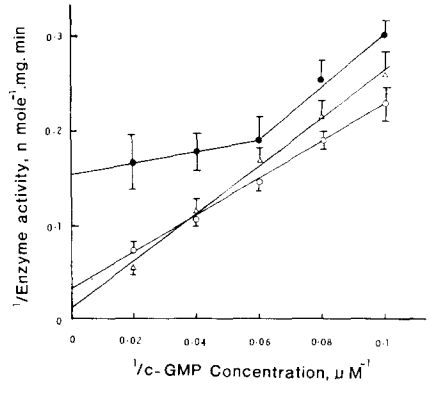

Fig. 3. Influence of change in cGMP concentration on the inhibitory action of DDT on the calmodulin-stimulated phosphodiesterase activity. Key: (△) control 344 nM calmodulin, (○) control 115 nM calmodulin, and (●) 344 nM calmodulin and 10^{-5} M DDT. See Fig. 1 for other conditions (method A).

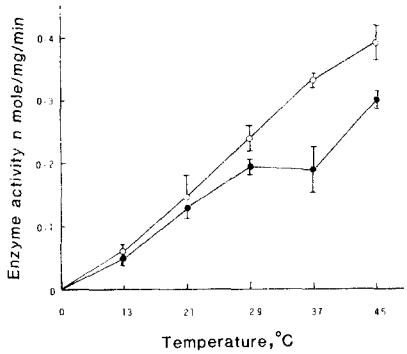

Fig. 4. Effect of temperature on calmodulin-stimulated phosphodiesterase activity in the presence (●) and absence (○) of 10^{-5} M DDT. See Fig. 1 for other conditions (method A). The level of calmodulin was 344 nM.

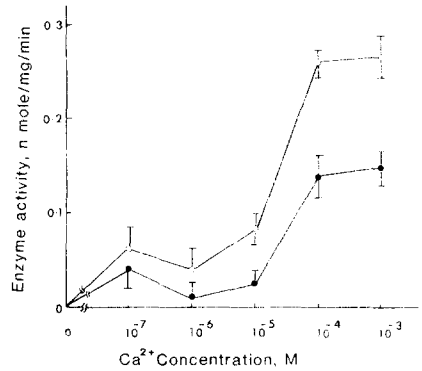

Fig. 5. Effect of Ca^{2+} concentration on the level of calmodulin-stimulated phosphodiesterase activity at 37°. The experiments were conducted in the presence (●) and absence (○) of 10^{-5} M DDT. See Fig. 1 for other conditions (method A).

acts with phosphodiesterase in a reversible manner (i.e. the (b) possibility above).

The same relationship holds in the case of the response of calmodulin–phosphodiesterase to DDT against temperature changes (Fig. 4). Here it is clearly shown that the effect of DDT was most pronounced at the optimum temperature for calmodulin action. DDT showed little inhibitory effect at a low temperature, where calmodulin was shown not to play a significant role in the case of mammalian systems. For instance, at 21° the extent of calmodulin stimulation of untreated enzyme was 5% (i.e. 0.093 vs 0.089), whereas that at 37° was 306% (Table 3). Another way to measure the significance of calmodulin inhibition is to test the effect of DDT inhibition at various Ca^{2+} concentrations. When the calcium concentration was varied (Fig. 5), the levels of DDT inhibition were observed to be apparent at all Ca^{2+} concentrations. It closely followed the Ca^{2+} activation curve obtained in the presence of calmodulin. The extents of DDT inhibition (i.e. % inhibition) were much higher at low Ca^{2+} concentrations ($Ca^{2+} \leq 10^{-5}$ M).

All these observations are consistent with the hypothesis that DDT affects only calmodulin and does not act on the phosphodiesterase proper in the concentration range tested. With this in mind, we tested the effects of various concentrations of DDT

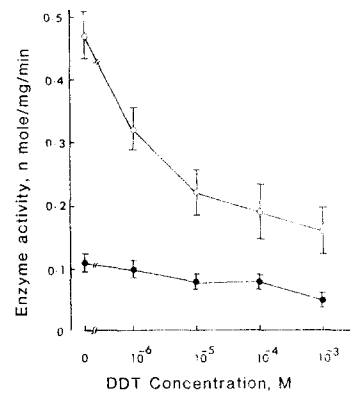

Fig. 6. Effect of DDT on the calmodulin-stimulated phosphodiesterase activity. Phosphodiesterase activity was determined in the absence (●) and presence (○) of 344 nM calmodulin. See Fig. 1 for other conditions (method A).

on phosphodiesterase both in the presence and absence of calmodulin (Fig. 6). In the absence of exogenously added calmodulin, DDT inhibition appeared to plateau at 10^{-5} to 10^{-4} M, whereas at 10^{-3} M there was a further decline in the enzyme

Table 3. Effects of various insecticides on the calmodulin-stimulated phosphodiesterase activity at two different temperatures*

Treatment	Enzyme activity (nmoles/mg/min)	
	21°	37°
None (= control)	0.089 ± 0.014	0.217 ± 0.007
+ Calmodulin	0.093 ± 0.013	0.880 ± 0.088
+ Calmodulin + permethrin	0.072 ± 0.016	0.238 ± 0.035
+ Calmodulin + deltamethrin	0.082 ± 0.012	0.618 ± 0.045
+ Calmodulin + allethrin	0.076 ± 0.012	0.631 ± 0.015
+ Calmodulin + cypermethrin	0.079 ± 0.006	0.618 ± 0.028
+ Calmodulin + DDT	0.078 ± 0.013	0.573 ± 0.004

* All insecticides were tested at 10^{-5} M. See Table 1 for other conditions. Data are expressed as mean ± S.D. of three to six determinations.

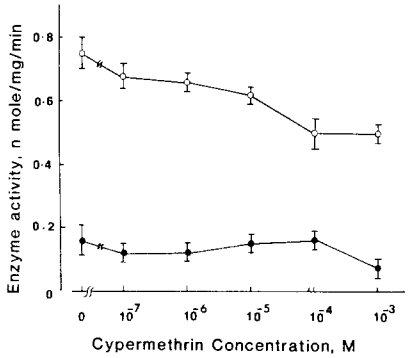

Fig. 7. Effect of cypermethrin on the calmodulin-stimulated phosphodiesterase activity. Phosphodiesterase activity was determined in the absence (●) and presence (○) 344 nM of calmodulin. See Fig. 1 for other conditions (method A).

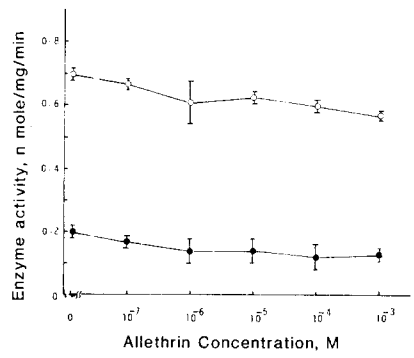

Fig. 9. Effect of allethrin on the calmodulin-stimulated phosphodiesterase activity. Phosphodiesterase activity was determined in the absence (●) and presence (○) of 344 nM. See Fig. 1 for other conditions (method A).

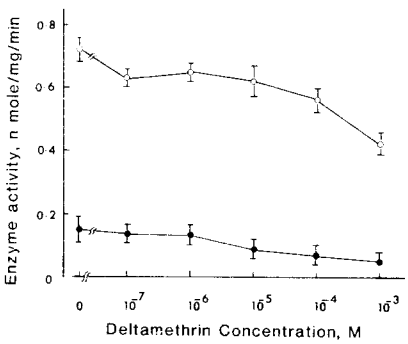

Fig. 8. Effect of deltamethrin on the calmodulin-stimulated phosphodiesterase activity. Phosphodiesterase activity was determined in the absence (●) and presence (○) of 344 nM calmodulin. See Fig. 1 for other conditions (method A).

activity. If one assumes that at 10^{-6} M the entire action of DDT is due to calmodulin interaction and that at 10^{-3} M some part of phosphodiesterase is also affected, one could estimate that about 26% of the activity of the original enzyme (i.e. without added calmodulin) was due to the endogenously present calmodulin. Similar tests were conducted with pyrethroid insecticides (Figs. 7–10). Based on the results, one may conclude that allethrin does not significantly affect calmodulin or phosphodiesterase. Deltamethrin at 10^{-5} M affected the enzyme part, while cypermethrin affected calmodulin at 10^{-4} M without affecting the enzyme. Permethrin was the most potent inhibitor affecting both calmodulin and the enzyme, particular at 10^{-4} M to 10^{-3} M. The above tendency may be confirmed by another approach to utilize the response of calmodulin stimulation to temperature. The results summarized in Table 3 clearly show that the inhibitory actions of these insecticides (except permethrin) are not significant at 21°, while at 37° all of their inhibitory actions were apparent in the presence of calmodulin.

Finally, to understand the stoichiometry of DDT inhibition of calmodulin, the amount of DDT bound to calmodulin under the standard assay condition at an approximate I_{50} concentration of DDT (i.e. 10^{-5} M) was determined. In the charcoal–dextran method, the level of DDT bound was determined to be 1.78 nmoles per 1 nmole of calmodulin and the in Sephadex G.75 column method it was 1.49 nmoles per 1 nmole of calmodulin.

DISCUSSION

Evidence has been provided in this work that DDT inhibits calmodulin, and that at concentrations below 10^{-4} M it does not inhibit phosphodiesterase. The apparent inhibition of the unfortified phosphodiesterase by DDT may be explained, if one assumes that approximately 20–50% of the enzyme activity observed is due to the native calmodulin present in the preparation. The fact that 10^{-4} M chlorpromazine could inhibit about 30% of the unfortified preparation (Table 2) supports the above view.

Calmodulin is a universal calcium binding protein. It is utilized in many Ca^{2+} requiring systems, and the list of calmodulin-requiring biochemical systems is increasing as our knowledge of its role is expanding [12]. Within the neural events calmodulin is known to participate in Ca^{2+}-Mg^{2+}-ATPase activities, both at presynaptic and postsynaptic sites, and probably the Na^{+}/Ca^{2+} exchange system both at phosphorylation and dephosphorylation steps [13]. Other important systems which are activated by calmodulin and therefore are potentially susceptible to inhibitory actions by these insecticides are, for example, cyclic nucleotide phosphodiesterase [14, 15], brain adenylate cyclase, brain membrane kinase [16], Ca^{2+}-Mg^{2+}-ATPase [17], myocin light chain kinase [18], calcium-dependent protein kinase located in synaptosomal membranes and synaptic vesicle [19], NAD kinase [20] and phosphorylase kinase [21]. There is an incipient indication that even the sodium channel activity is modified by exogenously added calmodulin and is inhibited by a specific calmodulin inhibitor, RO24571.* Therefore, there is a possibility that such a phenomenon of calmodulin inhibition could result in many deleterious consequences.

To understand the toxicological significance of such an interaction it is necessary to know the con-

* F. Matsumura and J. M. Clark, manuscript submitted for publication.

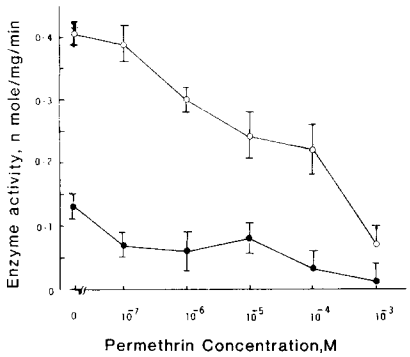

Fig. 10. Effect of permethrin on the calmodulin-stimulated phosphodiesterase activity. Phosphodiesterase activity was determined in the absence (●) and presence (○) of 344 nM calmodulin. See Fig. 1 for other conditions (method A).

centration of the inhibitor at the site in both *in vitro* and *in vivo* situations. Unfortunately, the true concentrations of these highly lipophilic and sparsely water-soluble chemicals at any biological target are very difficult to assess. Our data indicate that, at I_{50} concentration, 1.5 to 1.8 nmoles of DDT are bound to 1 nmole of calmodulin.

The concentration of calmodulin in the nervous system may vary from one source to another and also may be quite different from one species to another [16, 22]. One source [16] estimates it to be in the order of 5 μM in the brain. In that case, the DDT concentration to give I_{50} may be estimated to be 3.17 ppm. The level of DDT that has been frequently found in brains of DDT poisoned animals showing symptoms is in the order of 13–88 ppm [23]. While such data do not provide concrete evidence that DDT inhibits calmodulin *in vivo*, they indicate that *a priori* sufficient quantities of DDT are present to inhibit calmodulin in the brain of poisoned animals.

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REFERENCES

1. F. Matsumura, in *Pesticide Chemistry* (Eds. J. Miyamoto and P. C. Kearney), Vol. 3, p. 3. Pergamon Press, Oxford (1983).
2. F. Matsumura and J. M. Clark, *Prog. Neurobiol.* **18**, 231 (1982).
3. K. S. Sobue, S. Ichida, H. Yoshida, R. Yamazaki and S. Kakiuchi, *Fedn Eur. Biochem. Soc. Lett.* **99**, 199 (1979).
4. W. Y. Cheung, *Science* **207**, 18 (1980).
5. J. Hagman, *Fedn Eur. Biochem. Soc. Lett.* **143**, 52 (1982).
6. D. J. Wolff, P. G. Piorier, C. O. Brostrom and M. A. Brostom, *J. biol. Chem.* **252**, 4108 (1977).
7. N. Siegel and A. Haug, *Biochim. biophys. Acta* **744**, 36 (1983).
8. C. R. Caldwell and A. Haug, *Analyt. Biochem.* **116**, 325 (1981).
9. C. O. Brostrom and D. J. Wolff, *Archs Biochem. Biophys.* **172**, 30 (1976).
10. B. Weiss, W. Prozialeck, M. Cimino, M. S. Barnette and T. L. Wallace, *Ann. N.Y. Acad. Sci.* **356**, 319 (1980).
11. H. Linewaver and D. J. Burk, *J. Am. chem. Soc.* **56**, 658 (1934).
12. W. Y. Cheung, *Biochem. biophys. Res. Commun.* **38**, 533 (1970).
13. P. Caronii and E. Carafoli, *Eur. J. Biochem.* **132**, 451 (1983).
14. C. B. Klee, *Calcium and Cell Function* (Ed. W. Y. Cheung), Vol. 1, p. 58. Academic Press, New York (1980).
15. R. M. Levin and B. Weiss, *Molec. Pharmac.* **12**, 581 (1976).
16. C. B. Klee, T. H. Crouch and P. G. Richman, *A. Rev. Biochem.* **49**, 489 (1980).
17. H. Hidaka, T. Yamaki, M. Naka, H. Hayashi and R. Kobayashi, *Molec. Pharmac.* **17**, 66 (1980).
18. H. Hidaka, M. Naka and T. Yamaki, *Biochem. biophys. Res. Commun.* **90**, 694 (1979).
19. H. Shulman and P. Greengard, *Nature, Lond.* **271**, 478 (1978).
20. J. M. Anderson and M. J. Cormier, *Biochem. biophys. Res. Commun.* **84**, 595 (1978).
21. S. Shenolikar, P. T. W. Cohen, P. Cohen, A. C. Nairn and S. V. Perry, *Eur. J. Biochem.* **100**, 329 (1979).
22. M. N. Lin, *Molec. cell. Biochem.* **45**, 101 (1982).
23. W. J. Hayes, Jr., *Toxicology of Pesticides*, p. 167. Williams & Wilkins, Baltimore (1975).