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ROLE OF PHOSPHOLIPIDS IN THE INHIBITORY ACTION OF DDT AND PERMETHRIN ON THE NERVE ATPases OF LOBSTER, *HOMARUS AMERICANUS*

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Abstract—Efforts were made to understand the nature of the site of 1,1-bis-(*p*-chlorophenyl)-2,2,2-trichloroethane (DDT) inhibition of nerve ATPase. The phospholipid content of nerve preparations from the walking leg of the lobster was reduced by treating them with phospholipase A, or with a chloroform–methanol mixture at −75°. By these treatments the enzymes lost approximately 70 or 95% of their phospholipids and 50–80% of their Na,K- and Ca-ATPase activities. The lost ATPase activities could be partially restored by the addition of phospholipids, either the ones extracted from the lobster nerves or those from commercial sources. ATPase inhibition by DDT and permethrin was found to be highest in preparations where the phospholipids were removed by the above treatments, next highest with the untreated original enzyme, and least with the reconstituted ATPase regardless of the source of phospholipids used for reconstitution. This tendency was more pronounced in the case of Ca-ATPase. The effects of DDT and permethrin on inhibition of reconstituted Ca-ATPase were higher when the insecticide was first added to the protein portion and the enzyme was then reconstituted with the phospholipids, than when the same amount of insecticide was first added to the phospholipids which were then used for reconstitution.

The molecular basis of the interaction of DDT§ with the membrane constituents of the nervous system has long been debated. As early as 1955, Mullins [1] hypothesized that DDT fits into a molecular lattice of lipid molecules in the axonic membrane to cause characteristic nerve excitation. Holan [2] constructed the more elaborate hypothesis that DDT-type compounds fit into the Na^+ and K^+ channel by means of both protein and lipid binding. Tinsley *et al.* [3] and Hague *et al.* [4] took a direct approach and assessed the interaction of DDT with lecithin and found that indeed DDT binds with the polar end of the lecithin molecule. Matsumura and O'Brien [5] and O'Brien and Matsumura [6] were also able to show that DDT binds with certain nerve components of the central nervous system of the American cockroach. Because of the lipophilic property of DDT, it has been suggested that DDT owes its neurotoxicity to its ability to bind to the phospholipids and thereby disturb protein–phospholipid interactions [3, 4, 7, 8].

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§ Abbreviations: DDT, 1,1-bis-(*p*-chlorophenyl)-2,2,2-trichloroethane; permethrin, 3-(phenoxyphenyl)methyl (±)-*cis,trans*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-carboxylate; TCA, trichloroacetic acid; P_i, inorganic phosphate; EDTA, ethylene-diaminetetraacetic acid; EGTA, ethyleneglycol-bis-(β-amino-ethyl ether)*N,N'*-tetraacetic acid; ATP, adenosine triphosphate; BSA, bovine serum albumin; serine, L-α-phosphatidyl-L-serine; lecithin, L-α-phosphatidylcholine; and cephalin, L-α-phosphatidylethanolamine.

In the current investigation we have used the DDT-sensitive ATPases, (Ca-ATPase and Na,K-ATPase), of the lobster peripheral nerve [9–11] as a model system to study the interaction between phospholipids and DDT or permethrin. Permethrin is considered to be similar to DDT in its mode of action. We have in particular addressed the question of the role of phospholipids in the action of these insecticidal chemicals.

MATERIALS AND METHODS

Enzyme preparation

The procedure for the preparation of enzyme (cell membrane fraction) from peripheral nerves of walking legs of lobster (*Homarus americanus*) was identical to the method described earlier [10, 12].

In some experiments lobster nerve "axonic suspension" (800 g) or nerve homogenate was used as an enzyme source. The method of preparation of the axonic suspension has been described earlier [10, 11].

Phospholipase treatment

Lobster nerve preparations (20,000 *g* supernatant fraction) were treated with phospholipase A from *Vipera russelli.* For this purpose, 7 μl of phospholipase A (obtained in the form of a 50% glycerol suspension at a concentration of 2 mg/ml or 10 units/mg protein) was added to 0.8 ml of 100 mM Tris–HCl buffer at pH 7.1 containing 2 mM $CaCl_2$. The system was first heated at 100° for 3 min to destroy any proteases in the preparation and then was transferred to an ice bath. Upon cooling, 1 ml

of the enzyme source, 20,000 *g* crude supernatant fraction in 0.725 M sucrose, was added and the system was incubated at 30° for 10 min. The reaction was stopped by adding 0.2 ml of 100 mM Tris–HCl buffer (pH 7.1) containing 40 mM disodium EDTA. A 0.2 ml or 0.1 ml aliquot of the above digest containing a specified amount of protein, as described in the text, was used per ATPase assay or phosphorylation test. For controls an identically prepared enzyme source using 50% glycerol in place of phosphospholipase A was used.

Extraction of phospholipids

Phospholipid extraction was accomplished, using a methanol–chloroform (1:1) mixture at −75°, by adopting the cryobiochemical method [13, 14] as described below.

Lobster nerves were first cut into 0.1 mm sections using a tissue chopper and then were promptly dropped into a methanol–chloroform (1:1) mixture kept at −75° with dry ice and acetone. The nerve materials were then dispersed in the solvent at −75° using an omni-mixer (Sorvall) and were later homogenized in a Teflon–glass homogenizer at the low temperature (−75°). The homogenate was allowed to stand for an additional 1 hr at −75° (i.e. in a dry ice–acetone bath) to complete phospholipid extraction. A specially designed filtration system with a vacuum suction, similar to the one described by Freed and Sack [13], was used to keep the entire system at −75° and to remove the organic solvents. The phospholipid extracted nerve material left behind on the filter was washed once more with chilled chloroform–methanol mixture followed by chilled acetone. The resulting solid cake of nerve material was transferred to a cooled desiccator under vacuum and further held at refrigerating temperature to remove traces of solvents, if any. After complete drying, lipid extracted nerve tissue was brought to room temperature, and the enzyme solution was prepared using 0.5 to 2 mg of dry powder per ml in 0.725 M sucrose and 1 mM EDTA solution. A concentration of 20–230 μg protein/0.1 to 0.2 ml, as estimated by the method of Lowry *et al.* [15], was used.

To check the efficiency of extraction of phospholipid from lobster nerves using the above procedure, the amount of organic phosphates in phospholipids extracted from nerves was estimated using modified Fiske–Subbarrow's phosphorus assay mixture as described by Doherty and Matsumura [12]. The data obtained showed that 95.7% of total phospholipids was extracted by the above solvent extraction procedure, as evidenced by a second extraction of the same proteins which yielded only 4.3% of the phospholipids.

Assay methods

Na,K-*ATPase*. The colorimetric assay method for Na,K-ATPase has been described in detail earlier [9, 12, 16]. The standard assay mixture for Na,K-ATPase assay contained 60 mM NaCl, 60 mM KCl, 10 mM $MgCl_2$, 0.6 mM EGTA, 0.1 mM ouabain, and 30 mM Tris–HCl at pH 7.3. To a 1.6 ml aliquot of buffer, 0.2 ml of the enzyme was added at 0°. The reaction was started with the addition of 0.2 ml assay buffer containing 1 μmole ATP to give a final concentration of 5×10^{-4} M and was incubated at 30° for 30 min. At the end of the incubation, the reaction was stopped and the inorganic phosphate (P_i) produced was estimated colorimetrically at 620 nm.

Ca-*ATPase*. For the assay of Ca-ATPase, a radiometric charcoal method [10, 11] using [γ-^{32}P]ATP as a substrate was used. The ionic composition of the standard assay mixture was identical to the assay method for Na,K-ATPase except that it contained 0.8 mM Ca^{2+}, 0.5 mM EGTA, no Mg^{2+}, 0.1 mM ouabain, 2 mM KCN, and 0.1 mM EDTA (all expressed as final concentration). The total volume of assay mixture was 1 ml (i.e. 0.9 ml assay buffer and 0.1 ml enzyme). The amount of [^{32}P]ATP used was 8×10^{-8} M (final concentration). At the end of the incubation the [^{32}P]P_i liberated was estimated using the scintillation procedure as described earlier [11].

Reconstitution of enzyme

In some experiments reconstitution attempts were made. In such cases phospholipids (e.g. L-α-phosphatidyl-L-serine, L-α-phosphatidylethanolamine and L-α-phosphatidylcholine) were used. For this purpose, each phospholipid in quantities of 500 μg and 200 μg as optimum amounts for Na,K- and Ca-ATPase reconstitution, respectively, was transferred to assay tubes, either in chloroform or chloroform–methanol mixture, and the solvent was completely evaporated under nitrogen. To these tubes, the assay buffer (either for Na,K-ATPase or Ca-ATPase assay) and the enzyme preparations (either phospholipase A treated or untreated enzyme, or solvent extracted enzyme) were added and dispersed at 0° using a probe-type sonicator for 30 sec. The resulting enzyme solution was allowed to stand at 0° for at least 30 min to complete the reconstitution process. In some cases bovine serum albumin was also added to each tube before the addition of the inhibitors (DDT or permethrin, 10^{-5} M) and ATP to initiate the enzyme assays. The other experimental details for the ATPase assays were identical to those described above in assay methods.

Phosphorylation test

The assay method for phosphorylation of the terminal phosphate of ATP from [γ-^{32}P]ATP into lobster nerve protein was identical to the one described by Doherty and Matsumura [9, 12].

In some experiments phosphorylated proteins were resuspended in 2–3 ml chloroform–methanol mixture (4:1) and extracted for phospholipids. To achieve this, resuspended proteins were first held in the solvent mixture at room temperature for 30–45 min and then filtered through Whatman filter paper (No. 50 retains particles of 2.7 to 5 μm in size). The residual proteins in the tubes and on the filter paper were washed at least twice with 2–3 ml portions of chloroform–methanol mixture and filtered each time. The phospholipid extracted proteins were then dissolved in 0.5 ml of 0.2 N NaOH and counted for radioactivity. The filtrate comprising chloroform–methanol phospholipid extract was transferred to scintillation vials, evaporated completely under N_2,

dissolved in NaOH as above, and was also counted for radioactivity.

$^{45}Ca^{2+}$ uptake

Attempts have been made to study binding sites of Ca^{2+} in terms of enzyme protein and phospholipid portions in relation to the action of DDT. To assess this phenomenon, lobster nerve axonic suspension [11] was allowed to bind with $^{45}Ca^{2+}$ in the presence of 3 mM Na_2-ATP as described earlier. Following the binding of $^{45}Ca^{2+}$, the axonic suspension was extracted with chloroform–methanol (4:1) mixture for the phospholipids in a manner identical to that described above.

All assays were done in duplicate, and each experiment was repeated as indicated in the text; the data are expressed as the mean values. In some experiments, standard error (S.E.) was also calculated to express the extent of variation between the replicate and is indicated accordingly in the tables and figure.

$^{45}Ca^{2+}$ and [γ-^{32}P]ATP with high specific activities (15–30 mCi/mg and 20–35 mCi/mmole, respectively) were obtained from the New England Nuclear Corp., Boston, MA. Phospholipase A, phospholipids [phosphatidyl-L-serine (serine) 98%, L-α-phosphatidylethanolamine (cephalin) 95%, and L-α-phosphatidylcholine (lecithin) 98%], EDTA, EGTA, ouabain, Na_2-ATP and other biochemicals were obtained from the Sigma Chemical Co., St. Louis, MO. Other reagents were of analytical reagent grade with highest purity available, and deionized distilled water was used throughout the experiments. DDT was pure *p′,p′*-DDT with no contaminants as examined by gas–liquid chromatography (GLC) at the 1 ppm level. Permethrin (35% *cis*-, 65% *trans*-isomers) was provided by the FMC Corp., Middleport, NY.

RESULTS

Effects of phospholipase A on Na,K-*ATPases*

A preliminary experiment established that treatment of enzyme preparation with phospholipase A reduced the Na,K-ATPase activity to approximately 70% at a protein concentration of 28 μg/ml under our experimental conditions.

To reconstitute the ATPase, three different phospholipids were added separately to the crude supernatant fraction in the presence and absence of bovine serum albumin. The results (Table 1) are consistent with our previous observation that phospholipase A treatment reduces the ATPase activity. Furthermore, all three phospholipids were capable of restoring the Na, K-ATPase activity to a greater extent in the presence of albumin. In another experiment, the vulnerability of the reconstituted ATPase to DDT was examined by using L-α-phosphatidylethanolamine and phosphatidyl-L-serine (Table 2) in the presence of albumin. It was noted that phospholipase A treatment did not destroy the DDT sensitivity of the Na,K-ATPase.

Effects of chloroform–methanol extraction of Na,K- *and* Ca-*ATPase on DDT inhibition characteristics*

To ascertain that the above phospholipase A treatment was affecting only the phospholipid portions of the ATPases, a different approach via cryobiochemical extraction of phospholipids with a methanol–chloroform mixture was made. The resulting nerve powder from methanol–chloroform treatment was resuspended in the standard assay buffer for Na,K-ATPase and its DDT and ouabain sensitivities were assessed as before. A preliminary test with Na,K-ATPase indicated that the solvent-extracted enzyme had retained DDT sensitivity (50% inhibition at 10^{-5} M), as well as ouabain sensitivity (23% inhibition at 10^{-4} M). The addition of L-α-phosphatidyl-L-serine without albumin stimulated its activity by 35%. DDT at the same concentration inhibited this reconstituted enzyme by 38.5%.

In contrast to the case of Na,K-ATPase, the Ca-ATPase activity was greatly reduced by extraction of phospholipids from lobster nerve. Also, the degree of restoration of the enzyme activity by reconstitution with phospholipids and albumin was relatively low in the case of Ca-ATPase (Table 3).

Table 1. Effect of addition of certain phospholipids to phospholipase A treated enzyme preparation of lobster nerves at 30°*

	Na,K-ATPase			
	No albumin		Plus albumin	
Treatment	P_i (μmoles/tube) per 30 min	% Activity	P_i (μmoles/tube) per 30 min	% Activity
Control	0.260	100	0.182	100
Phospholipase A treated enzyme	0.120	46	0.115	63
Treated enzyme + L-α-phosphatidylcholine	0.115	44	0.190	104
Treated enzyme + L-α-phosphatidyl-L-serine	0.105	40	0.180	99
Treated enzyme + L-α-phosphatidylethanolamine	0.165	63	0.300	165

* Each tube contained 50 μg enzyme protein in the presence or absence of 1.6 mg bovine serum albumin added prior to ATP addition to start the reaction. The amount of each phospholipid used for reconstitution was 500 μg/assay tube as specified in Materials and Methods.

Table 2. Effect of DDT in Na,K-ATPase activities of the crude supernatant fraction from lobster nerve preparation at 30°

Treatment*	ATPase			
	P_i (μmoles/tube) per 30 min	% Activity	P_i (μmoles/tube) per 30 min	% Activity
Control experiment				
Control (non-treated)	0.135	100	0.155	100
+ DDT (10^{-15} M)	0.080	59	0.102	66
	L-α-Phosphatidylethanolamine for reconstitution		L-α-phosphatidyl-L-serine for reconstitution	
Untreated enzyme source				
+ Phospholipid†	0.192	142	0.170	110
+ DDT + phospholipid†	0.187	139	0.158	102
Enzyme source treated with phospholipase A				
Control (treated)	0.060	44	0.087	56
+ DDT (10^{-5} M)	0.028	21	0.047	30
+ Phospholipid†	0.108	80	0.135	87
+ DDT + phospholipid†	0.070	54	0.107	69

* In the presence of bovine serum albumin (1.6 mg/tube) and 28 μg enzyme protein.

† The phospholipids used for reconstitution were either L-α-phosphatidylethanolamine or L-α-phosphatidyl-L-serine, added in 500 μg quantity/assay tube. DDT as specific inhibitor was added in 10 μl ethanol, while an equivalent amount of ethanol was added to the controls and incubated at room temperature for 10 min before the start of ATPase assay.

Nevertheless, the data clearly indicated that susceptibility of the enzyme to inhibitors (DDT and permethrin) was not reduced. Rather, phospholipid extraction increased the inhibitory effect of DDT and permethrin by 22–40%, respectively, over the effect observed using the unextracted nerve preparation. When the phospholipid extracted Ca-ATPase was reconstituted using phospholipids from lobster nerve, the overall enzyme activity increased but the inhibitory effects of DDT and permethrin were greatly reduced (Table 3).

Attempts were also made to reconstitute the Ca-ATPase using three different commercially available phospholipids separately, and the vulnerability of such reconstituted enzyme DDT and permethrin was also examined. L-α-Phosphatidyl-L-serine was found to increase the Ca-ATPase activity, but the other two phospholipids (L-α-phosphatidylethanolamine and L-α-phosphatidylcholine) were ineffective (Table 4). Also, as observed with lobster phospholipids, Ca-ATPase, reconstituted with either of the commercial phospholipids, was found to be less sensitive to DDT and permethrin than phospholipid extracted enzyme.

The potencies of DDT and permethrin toward the phospholipid extracted Ca-ATPase were also studied at various temperatures (Fig. 1). When percentages of inhibition were calculated, both DDT and permethrin were found to be potent inhibitors at lower temperatures (8° and 15°). The result indicates that

Table 3. Sensitivities of lobster nerve homogenate, methanol–chloroform extracted, and reconstituted Ca-ATPase to DDT and permethrin at 30°

Enzyme source*	Ca-ATPase†		
	Control	DDT (10^{-5} M)	Permethrin (10^{-5} M)
Nerve homogenate	2515.33 ± 148.94	1534.44 ± 95.92	2200.40 ± 45.23
Solvent extracted enzyme	219.45 ± 21.56	84.40 ± 10.40	84.81 ± 10.69
+ Lobster phospholipids	374.10 ± 11.36	284.51 ± 21.97	308.03 ± 33.58
+ Lobster phospholipids + albumin‡	381.48 ± 81.70	240.28 ± 19.48	208.03 ± 34.56

* In assay with nerve homogenates, 18.7 μg protein/assay tube was used while the phospholipid extracted concentration was 50 μg dry powder, equivalent to 18–20 μg protein/assay tube as estimated by the method of Lowry *et al.* As outlined in Materials and Methods, reconstitution of the enzyme was carried out by using 0.2 ml equivalent of chloroform solution of phospholipids previously extracted from lobster nerves. The solvent was evaporated under N_2 before being reconstituted with the enzyme protein. DDT and permethrin as specific inhibitors were added in 10 μl ethanol and incubated for 10 min at room temperature before the start of reaction.

† Ca-ATPase was assayed as pmoles $[^{32}P]P_i$ liberated per mg per 10 min. See Materials and Methods for details. Values are the means of three to four independent experiments carried out in duplicate ± S.E.

‡ BSA (1.6 mg/tube).

Table 4. Effect of different phospholipids on reconstitution of methanol–chloroform extracted Ca-ATPase and their sensitivities to DDT and permethrin at 30°

Enzyme source*	Ca-ATPase† Control	DDT (10^{-5} M)	Permethrin (10^{-5} M)
Solvent extracted enzyme	280.13 ± 18.77	139.77 ± 31.74	131.57 ± 16.47
+ L-α-Phosphatidyl-L-serine	424.34 ± 18.62	368.36 ± 26.46	311.72 ± 8.13
+ L-α-Phosphatidyl-choline	283.57 ± 22.32	220.26 ± 29.85	228.51 ± 27.45
+ L-α-Phosphatidyl-ethanolamine	219.59 ± 22.48	177.38 ± 17.03	187.80 ± 2.85

* Each assay tube contained 20 μg protein as estimated by the method of Lowry *et al.* Reconstitution of the enzyme with the phospholipids was effected by adding 200 μg of each phospholipid to the tube. Solvent unextracted enzyme activity and its sensitivity toward DDT and permethrin are shown in Table 3.

† Ca-ATPase was assayed as pmoles [^{32}P]P_i liberated per mg per 10 min. See Materials and Methods for details. Values are averages of two independent experiments done in duplicate ± S.E.

the Ca-ATPase was identical to the one studied by Ghiasuddin and Matsumura [11], and that even after phospholipid extraction it behaved in a qualitatively identical manner as to temperature changes.

Attempts were then made to study whether the order of the addition of the inhibitor, either first to the phospholipids or to the phospholipid extracted enzyme protein in reconstitution, would make any difference in the sensitivity of Ca-ATPase to DDT and permethrin. For this purpose, an experiment was designed in which either the enzyme proteins or the phospholipids were pretreated with the inhibitor (i.e. DDT or permethrin) at 15° and the enzyme was reconstituted as described above using corresponding untreated phospholipid or enzyme protein, respectively. Under such an assay method, inhibition of Ca-ATPase by both DDT and permethrin was found to be greater when proteins were first treated and reconstituted with untreated phospholipids, as compared to the cases where the inhibitors were first added to phospholipids and reconstituted with the untreated proteins (Table 5).

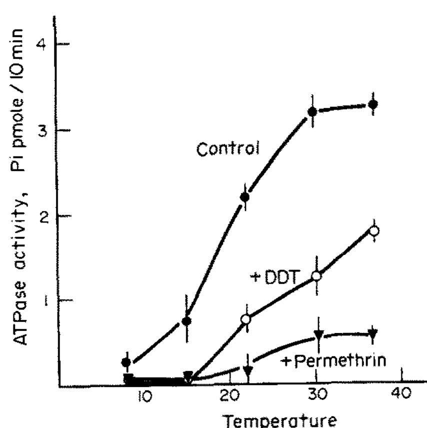

Fig. 1. Ca-ATPase activity of solvent extracted enzyme and its sensitivity to DDT (10^{-5} M) and permethrin (10^{-5} M) at different temperatures, under standard protein (18.7 μg/tube) and assay conditions.

Role of phospholipids in phosphorylation activities of Ca-ATPase

To further confirm and substantiate the above phenomenon, another set of experiments was conducted using phosphorylation as the criterion. Phosphorylation of lobster nerve enzyme proteins was carried out using the 90,000 *g* lobster nerve membrane fraction and γ-^{32}P-labeled ATP. Preliminary experiments showed that in the presence of Ca^{2+} and the absence of Mg^{2+} there was almost no phosphorylation. This was in agreement with the findings that Mg^{2+} stimulates, while Ca^{2+} inhibits, phosphorylation by the nerve ATPases of the lobster nerve [9, 12]. Therefore, the phosphorylation experiment was carried out using 3 mM Mg^{2+} under the standard assay conditions as described before [9, 12]. As observed earlier, there was an increase in phosphorylation of the enzyme when DDT was present in the system (Table 6). Furthermore, when the phosphorylated enzyme preparation was divided into phospholipid and protein fractions, a greater portion (75–80%) of the ^{32}P incorporated was found to partition into the protein part of the enzyme, while only 16% and 20% partitioned into the phospholipid portion in DDT-treated and untreated enzyme preparations respectively (Table 6).

Role of phospholipids in $^{45}Ca^{2+}$ binding

The results of the test on $^{45}Ca^{2+}$ uptake by lobster nerve preparation are given in Table 7. In agreement with earlier findings [10, 11], DDT was found to inhibit the process of $^{45}Ca^{2+}$ binding by lobster nerve in the presence of ATP. Extraction of the phospholipids from $^{45}Ca^{2+}$ loaded nerve (axonic suspension) with chloroform and methanol showed that almost 99% of the bound $^{45}Ca^{2+}$ was in the protein part of the nerve and only a negligible amount was found to be associated with the phospholipid portion (Table 7).

DISCUSSION

As for the nature of DDT interactions with phospholipids and the ATPases, Sharp *et al.* [8] have

Table 5. Pretreatment of enzyme protein or phospholipids with DDT and permethrin and their effects on the sensitivity of reconstituted Ca-ATPase at 15°

Enzyme source*	Ca-ATPase† Control	DDT (10^{-5} M)	Permethrin (10^{-5} M)
Solvent extracted enzyme	136.45 ± 68.40	6.28 1.42	25.90 ± 6.50
Inhibitors‡ added first to enzyme protein and reconstituted with lobster phospholipids	197.54 ± 90.81	84.11 ± 42.17	51.82 ± 36.60
Inhibitors‡ added first to lobster phospholipids and reconstituted with enzyme protein	200.87 ± 89.90	144.21 ± 68.02	50.78 ± 31.21
Inhibitors‡ added first to enzyme protein and reconstituted with L-α-phosphatidyl-L-serine	404.13 ± 34.55	161.11 ± 50.26	72.56 ± 25.90
Inhibitors‡ added first to L-α-phosphatidyl-L-serine and reconstituted with enzyme protein	491.43 ± 75.63	262.91 ± 102.17	223.31 ± 104.70

* The concentrations of proteins and phospholipids were similar to those in Tables 6 and 7.

† Ca-ATPase was assayed as pmoles [^{32}P]P_i liberated per mg per 10 min. See Materials and Methods for details. Values are means of three to five independent experiments done in duplicate ± S.E.

‡ DDT or permethrin was added in 10 μl ethanol.

Table 6. Role of phospholipids in phosphorylation of lobster nerve membrane fraction from γ-labeled [^{32}P]ATP in the presence and absence of DDT at 30°

Treatments*	^{32}P-Incorporation† [pmoles·mg^{-1}·(4 min)$^{-1}$] Membrane protein fraction	Membrane phospholipid fraction
Nerve membrane fraction		
Control	0.836 ± 0.016	
DDT-treated (10^{-5} M)	1.437 ± 0.047	
Nerve membrane protein and phospholipid fractions		
Control	0.819 ± 0.030	0.170 ± 0.012
DDT-treated (10^{-5} M)	1.419 ± 0.061	0.305 ± 0.013

* Protein concentration was 100 μg/assay tube.

† Values are means of two independent experiments done in duplicate ± S.E.

Table 7. DDT effect on $^{45}Ca^{2+}$ binding by protein and phospholipid portions of lobster nerve

Treatments*	$^{45}Ca^{2+}$ uptake† [nmoles·mg^{-1}·(10 min)$^{-1}$] Protein fraction	Phospholipid fraction
Axonic suspension		
Control	4.914 ± 1.493	
DDT-treated (10^{-5} M)	2.388 ± 0.741	
Axonic nerve protein and phospholipid		
Control	3.442 ± 0.425	0.025 ± 0.013
DDT-treated (10^{-5} M)	1.476 ± 0.426	0.022 ± 0.003

* Protein concentration was 50 μg/assay tube.

† Values are means of two experiments done in duplicate ± S.E.

studied the effect of added phospholipids on the inhibitory behavior of DDT on Na,K-ATPase from beef brain and kidney. They found that acidic phospholipids, particularly L-α-phosphatidyl-L-serine, partially reversed the inhibition of DDT. Based on this, they postulated that the action of DDT on these enzymes depends on its ability to noncompetitively interact with the protein–phospholipid association. If such is the case, the level of DDT inhibition should become high when phospholipids are added to the lipid-deficient enzymes. This is exactly the opposite of what we observed here. A number of workers have also shown that DDT binds with phospholipids [3, 4], and there have been suggestions to relate the above phenomenon to the action mechanism of DDT. However, if the major site of attack of these insecticides should be on phospholipids, the addition of these chemicals to phospholipids before the reconstitution (see Table 5) would have induced an increased level of inhibition of the reconstituted Ca-ATPase.

It seems logical, therefore, to assume that the bulk of phospholipids present in normal enzyme preparation has nothing to do with DDT inhibition of ATPases. However, they may have significant DDT-binding capability [3, 4] and, as such, act as competitors for the insecticide binding to the actual target site on the protein part. According to this hypothesis, many of the DDT-sensitive sites of the ATPases must be exposed in the phospholipid delipidated enzyme preparations, making them more sensitive to the attack of DDT. When a sufficient amount of phospholipids is added to the above system, a significant portion of DDT is taken up by the phospholipids, and thereby the portion of DDT available to the ATPases becomes less. If such is the case, the reduction in percent inhibition of the ATPase by DDT with the addition of phospholipids found by both Sharp *et al.* [8] and us in this work can be well explained. This view is substantiated by the fact that DDT or permethrin added to phospholipids before the reconstitution of Ca-ATPase was less inhibitory than in the cases where the protein part of the ATPase was treated first with DDT or permethrin before the addition of phospholipids to reconstitute the enzyme (Table 5). Additional supporting evidence may be found in the data derived from our current experiments with respect to $^{45}Ca^{2+}$ binding behavior. Almost 99% of the $^{45}Ca^{2+}$ binding in the protein part of the enzyme rather than in the lipid portion (Table 7), and furthermore DDT significantly inhibits the $^{45}Ca^{2+}$ binding process of the protein part of the ATPase.

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