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Plasma Membrane ATPases from Various Tissues of the Cockroach *(Periplaneta americana)* and Mouse Influenced by Toxaphene[1]

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Toxaphene® (chlorinated camphene with a 67-69% chlorine content; average empirical formula $C_{10}H_{10}Cl_8$) has been one of the least tested of the chlorinated insecticides on adenosine triphosphatase (ATPase) activities. DESAIAH and KOCH (1975) investigated the effects of toxaphene on the ATPase activities of kidney, brain and gill tissues from the catfish, Ictalurus punctatus. Toxaphene inhibited all 3 ATPases in all 3 tissues tested. Effects of toxaphene on ATPase of kidney, brain and liver of the mouse both in vitro and in vivo were studied by TROTTMAN and DESAIAH (1978). Na^+K^+ ATPase in brain and kidney was inhibited by toxaphene in vitro with an estimated IC_{50} of 7.5 μM. Oligomycin-sensitive (O-S) Mg^{2+} ATPase was inhibited in vitro in a dose dependent manner in kidney, brain and liver with IC_{50} values of 15, 15 and 20 μM, respectively. Oligomycin-insensitive (O-I) Mg^{2+} ATPase showed less sensitivity to toxaphene as compared to (O-S) Mg^{2+} ATPase in all tissues examined. For in vivo effects mice were fed with toxaphene in corn oil at 10, 25, and 50 mg/kg/day for 3 days; animals were sacrificed 36 h after the last treatment. ATPase activities of kidney and liver were decreased in toxaphene-treated mice while brain ATPases were not affected. The authors suggested that this insecticide may be interfering with hepatic and renal functions rather than nervous functions.

Our objectives were to test toxaphene in vivo and in vitro on the plasma membrane ATPases of kidney, brain and liver of the mouse, and in vitro on the central nervous system (CNS) and the Malpighian tubules of the cockroach. The results could aid in elucidating the mode of action of toxaphene.

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METHODS AND MATERIALS

Male Swiss Webster mice were used at 9-11 weeks of age and weighing 27.71 ± 0.13 g. They had free access to Wayne's Lablox® and water. Thirty five mice, each constituting a replicate, were administered by oral gavage an LD_{50} dose of 112 mg/kg of toxaphene (obtained from Hercules, Inc. batch number X16189-49) in 0.16 cc of Mazola® corn oil. Mice surviving the dose were sacrificed 60-90 min later, i.e. during the second poisoning symptom period as described by BUTLER and CROWDER (1977). If death occurred within the first hour, mice were immediately processed. Controls (N = 18, 1 mouse/replicate) were dosed with 0.16 cc corn oil and sacrificed 60-90 min later.

Twenty mice (each constituting a replicate) were used to study the effects of *in vitro* toxaphene exposure on mouse tissues. Each tissue was subjected to 4 treatments and 2 controls: 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} M toxaphene; 10 µL ethanol and untreated controls. Three subsamples were run for each treatment in each of the 3 tissues.

Adult male cockroaches, *Periplaneta americana* (L.), were maintained in a rearing room (24 ± 3°C, 40% R.H., and a light: dark cycle of 9:15 h with free access to water and food consisting of honey and glycerin-coated Purina® dog chow (1:1:12 v/v). Tissues from 3 cockroaches were combined to constitute a replicate. Fourteen replicates were performed in the manner described above for *in vitro* exposure to mice.

Upon sacrificing a mouse, brain, whole liver, and right kidney were dissected, washed with ice cold homogenization solution (pH 7.6) containing 0.25 M sucrose, 10 mM Tris and 0.5 mM EDTA (CLARK and NICKLAS 1970), and cut into small pieces with scissors. Each tissue was then transferred to a pre-weighed beaker containing 2 mL ice cold homogenization solution and weighed. Malpighian tubules and CNS were dissected from adult cockroaches and transferred immediately to ice cold homogenization solutions.

The method used in tissue fractionation was after KOCH (1969) except for modification of the homogenization solution previously described. All tissues were homogenized by a motor-driven homogenizer at 500 rpm for 2 min. The liver was homogenized in 15 mL homogenization solution, brain and kidney in 10 mL each, and cockroach tissues in 5 mL each. The whole homogenate was centrifuged for 10 min, with 2 washings, at 0°C and 900 g in a refrigerated centrifuge. The supernatant from cockroach tissues was retained as the homogenate fraction. Mouse tissues were further centrifuged for 20 min at 0°C and 13,000 g; the resulting pellet was resuspended in homogenization solution to a volume of 5 mL and used as the homogenate fraction. Glassware used in the fractionation procedure was maintained ice cold with crushed ice.

The reaction mixture was prepared as a stock solution and kept refrigerated. It contained (as final concentrations in the stock solution) 5 mM ATP (adenosine 5'-tri-phosphate disodium salt; Eastman Kodak Co.), 5 mM Mg^{2+} (as Cl^- salt; Merck Chemical Co.),

90 mM Na^+, 19 mM K^+ (both Na^+ and K^+ as Cl^- salts; Matheson Coleman and Bell Manufacturing Chemists), 120 mM imidazole buffer (pH 7.5; J. T. Baker Co.). Another stock reaction mixture was prepared as above except for the addition of 1 mM ouabain (ICN Pharmaceuticals, Inc.). One hundred microliters of each homogenate fraction were added to 3 mL of reaction mixture. Enzyme activities were measured at 37°C for 40 min. The reaction was stopped by addition of 200 µL ice cold 30% TCA.

The reaction mixture used in in vitro experiments was identical with that of the in vivo experiment except for the addition of toxaphene. Toxaphene stock solutions were prepared in ethanol in such a way that when added to 10 µL aliquots they would give a final concentration of 10^{-4}, 10^{-5}, 10^{-6} or 10^{-7} M toxaphene in 3 mL reaction mixture.

Mg^{2+}-ATPase activity was measured in the presence of ouabain in the reaction mixture (McILWAIN 1963). Na^+K^+ ATPase activity was calculated as the difference between total ATPase and Mg^{2+}-ATPase measured activities. ATPase activities were obtained in terms of quantities of inorganic phosphate (Pi) released during the reaction. One milliliter of the sample was treated according to the method of OHNISHI et al. (1975) for ATPases and read at 750 µm in a spectrophotometer. Samples of homogenate fractions were treated according to the method of LOWRY et al. (1951) and read at 750 µm. Protein concentrations of the samples were determined from a standard curve prepared from bovine albumin (Sigma Chemical Co.).

RESULTS

In Vivo Experiment:

Kidney ATPases were more sensitive to toxaphene than others. Na^+K^+ ATPase was inhibited significantly only in the kidney, and the Mg^{2+} ATPase was inhibited significantly in all 3 tissues (Table 1). The highest inhibition percentage was observed in the kidney for both Na^+K^+ and Mg^{2+} ATPases, 12.6 and 15.0%, respectively. In general Mg^{2+} ATPase was more sensitive to toxaphene than the Na^+K^+ ATPase in all tissues.

In Vitro Experiment:

Mouse - The solvent control (10 µL ethanol) reduced the activity significantly in all ATPases tested except the Na^+K^+ ATPase of the brain, and displayed an insignificant stimulation of the liver Mg^{2+} ATPase (Table 1). The highest inhibition due to ethanol was in the Na^+K^+ ATPase of liver (10.9%) and the only stimulation was a 2.2% increase in Mg^{2+} ATPase of the same tissue.

Na^+K^+ ATPase was significantly inhibited only in the kidney homogenates by all concentrations of toxaphene when compared to either of the controls. The highest concentration (10^{-4}M) produced the highest degree of inhibition and vice versa. The maximum inhibition observed was 44.0%. This activity was not inhibited in the brain homogenates when compared to both controls. In liver homogenates this enzyme activity displayed

TABLE 1

Na^+K^+ ATPase and Mg^{2+} ATPase specific activities in kidney, brain and liver from toxaphene-dosed mice, *in vivo*, and from tissue homogenates subjected *in vitro* to various concentrations of toxaphene. -- Mean (± S.E.) specific activity expressed in μM Pi/mg protein/h.

	Kidney		Brain		Liver	
	Na^+K^+ ATPase	Mg^{2+} ATPase	Na^+K^+ ATPase	Mg^{2+} ATPase	Na^+K^+ ATPase	Mg^{2+} ATPase
In vivo Experiment:						
Control (corn oil)	36.3 ± 1.0b[1/]	47.3 ± 0.7b	16.8 ± 0.4a	21.2 ± 0.3b	4.2 ± 0.1a	10.0 ± 0.3b
Toxaphene (LD_{50})[2/]	31.7 ± 0.5a	40.2 ± 0.6a	16.7 ± 0.3a	19.3 ± 0.3a	4.1 ± 0.1a	9.0 ± 0.2a
In vitro Experiment:						
Controls:						
Untreated	36.7 ± 0.7e	48.2 ± 0.5d	16.9 ± 0.3a	21.1 ± 0.2d	4.3 ± 0.1be	10.2 ± 0.2b
Solvent[3/]	34.8 ± 0.6d	45.1 ± 0.5c	16.9 ± 0.2a	20.0 ± 0.3b	3.9 ± 0.1a	10.4 ± 0.1b
Toxaphene (M):						
10^{-4}	20.6 ± 0.6a	40.3 ± 0.6a	16.3 ± 0.4a	19.5 ± 0.2a	4.0 ± 0.1ac	8.4 ± 0.2a
10^{-5}	27.7 ± 0.4b	40.2 ± 0.5a	16.3 ± 0.2a	20.6 ± 0.3c	4.0 ± 0.1ac	10.1 ± 0.1b
10^{-6}	27.7 ± 0.3b	42.6 ± 0.3b	16.4 ± 0.1a	20.4 ± 0.3c	3.8 ± 0.1a	10.1 ± 0.2b
10^{-7}	32.5 ± 0.6c	42.8 ± 0.3b	16.8 ± 0.2a	20.5 ± 0.3c	4.5 ± 0.1b	10.3 ± 0.1b

1/ Means followed by the same letter in each tissue within an experiment are not significantly different (LSD, $\alpha = 0.05$)

2/ 112 mg/kg toxaphene

3/ 10 μL ethanol

variability in response to toxaphene with a significant inhibition caused by 10^{-6}M, whereas the higher concentrations (10^{-4} and 10^{-5}M) inflicted no significant reduction.

Mg^{2+} ATPase was significantly reduced by all toxaphene concentrations in both the kidney and the brain where the highest dose demonstrated the highest degree of inhibition. However, in the kidney the higher concentrations (10^{-4} and 10^{-5}M) were equipotent and the lower concentrations (10^{-6} and 10^{-7}M) were equipotent; in the brain the 3 lower concentrations (10^{-5}, 10^{-6} and 10^{-7}M) had the same effect. In the liver homogenates only the highest concentration exhibited a significant reduction in Mg^{2+} ATPase compared to both controls. Total ATPase was inhibited 28.9%, 6.0%, and 14.7% in kidney, brain, and liver, respectively, by the highest concentration which gave the highest degree of inhibition.

P. americana - The solvent control inhibited activity in Na^+K^+ ATPase of the CNS (Table 2). Otherwise, it did not have a significant effect with the exception of the total and the Mg^{2+} ATPase of the Malpighian tubules which showed a slight stimulation that reached 9.6% in the Mg^{2+} ATPase activity.

Na^+K^+ ATPase was significantly reduced by all toxaphene concentrations used in the CNS. The 10^{-4} concentration gave the highest inhibition (58.9%); 10^{-5} and 10^{-6}M were equipotent giving 48.7 % and 48.4%, respectively; and the lowest concentration (10^{-7}M) gave 17.9% inhibition. In Malpighian tubules, Na^+K^+ ATPase was significantly reduced by 10^{-4} and 10^{-5}, was not affected by 10^{-6}, and was significantly stimulated by 10^{-7}M.

Mg^{2+} ATPase of the CNS was reduced by 10^{-4} and 10^{-5}M. The lower concentrations (10^{-6} and 10^{-7}M) had no effect. In Malpighian tubules, Mg^{2+} ATPase was not inhibited but showed a trend of stimulation which reached statistical significance at the lowest concentration (10^{-7}M) when compared to the untreated control. However, the initial increase in this activity in this tissue was as high as 9.6% due to ethanol alone. The higher dose (10^{-4}M) inflicted a significant reduction on the stimulation by ethanol. The lower doses were unable to nullify the ethanol effect.

Regressions:

Two regression analyses were conducted to determine the significance of the dose response of each ATPase activity in each tissue of both animals. The linear regression included the solvent treatment (ethanol) as the 0 toxaphene treatment, but the linear $\log_{10}$ regression was made for the 4 toxaphene concentrations only. The linear regression indicated that there was a dose response in all ATPases of the kidney, all ATPases of the Malpighian tubules, total and Na^+K^+ ATPases of the CNS and the Na^+K^+ ATPase of the liver. With linear $\log_{10}$ regressions, where data for control treatments were omitted, only the Na^+K^+ ATPase of the brain did not show a statistically significant dose response to toxaphene.

TABLE 2

Na^+K^+ and Mg^{2+} ATPase specific activities in homogenates of P. americana CNS and Malpighian tubules subjected in vitro to various concentrations of toxaphene. -- Mean (± S.E.) specific activity expressed in μM Pi/mg protein/h.

	CNS		Malpighian tubules	
	Na^+K^+ ATPase	Mg^{2+} ATPase	Na^+K^+ ATPase	Mg^{2+} ATPase
Controls				
Untreated	73.4 ± 2.1e[1/]	40.1 ± 1.9c	48.3 ± 1.9c	48.8 ± 1.5a
Solvent[2/]	63.1 ± 1.7d	39.3 ± 1.3c	46.0 ± 1.4c	53.5 ± 1.6ab
Toxaphene (M)				
10^{-4}	30.2 ± 0.6a	28.4 ± 1.1a	22.9 ± 0.9a	48.9 ± 1.5a
10^{-5}	37.7 ± 1.0b	36.8 ± 1.3b	27.8 ± 1.4b	50.7 ± 1.2ab
10^{-6}	37.9 ± 1.6b	37.4 ± 1.2bc	46.7 ± 1.2c	53.3 ± 1.4ab
10^{-7}	60.2 ± 1.5c	38.8 ± 1.3bc	52.3 ± 1.4d	55.2 ± 1.6b

[1/] Means followed by the same letter in each tissue are not significantly different (LSD, $\alpha = 0.05$)

[2/] 10 μL ethanol

DISCUSSION

ATPase activity of the plasma membrane-rich nitochondrial fraction from mouse liver was extremely low. The protein content of the samples from the liver fractions was very high, resulting in the lowest specific activity encountered in spite of the fact that these homogenates gave the highest Pi spectrophotometric readings. The small variations in specific activities were exaggerated when transferred to percentage inhibition due to these low specific activity values. Electron microscopy seemed to provide an explanation for the liver homogenate behavior. Electron microscopy was carried out to insure that the homogenate fractions were rich in plasma membrane vesicles. Electron micrographs of the liver fraction revealed the presence of blood cells and cell debris in large quantities compared to plasma membrane vesicles, which resulted in the high protein content, thus, lowering the specific activity. Electron micrographs of kidney and brain fractions demonstrated a resemblance to fraction β in these tissues as presented by KOCH (1969).

Ethanol was reported to have no effect on ATPase activities when used as a solvent for pesticides at 10 μL or less per 3 mL assay reaction mixture (DESAIAH and KOCH 1977). But ethanol at this quantity caused reduction in most of the ATPase activities tested in our *in vitro* experiments. However, Mg^{2+} ATPase activity of the Malpighian tubules exhibited a stimulation that reached 9.6%. The highest toxaphene concentration used (10^{-4}M) gave an increase of 0.1%; this means, if not for the initial stimulation by ethanol, toxaphene might have displayed inhibition.

In vivo, an LD_{50} dose of toxaphene inhibited the Na^+K^+ ATPase in the kidney, and the Mg^{2+} ATPase in all 3 mouse tissues tested. But this dose did not cause a 50% inhibition of activity in any of the ATPases tested.

TROTTMAN and DESAIAH (1978) reported inhibition of Na^+K^+ ATPase in both liver and kidney with 15, 25 and 50 mg/kg/day of toxaphene administered for 3 days. Mice were sacrificed 36 hrs after last application. However, in our study mice were sacrificed not later than 90 min after administration if death had not occurred. The inhibition of Mg^{2+} ATPase of kidney and liver reported by TROTTMAN and DESAIAH is in agreement with results obtained herein. However, they were unable to detect inhibition in brain Mg^{2+} ATPase. *In vitro* administration of toxaphene was reported by TROTTMAN and DESAIAH to have inhibitory effects on Na^+K^+ ATPase activity of mouse brain, kidney and liver with an IC_{50} value of 7.5 μM toxaphene. In our study the highest inhibition obtained was 44.0% in Na^+K^+ ATPase of kidney caused by 10^{-4}M. As inhibition did not reach 50%, IC_{50} values were not calculated. Our study agreed with theirs that toxaphene had no effect *in vivo* on brain Na^+K^+ ATPase and that those of the kidney are the most sensitive ATPases to toxaphene inhibition. Further agreement was the *in vivo* toxaphene inhibition of kidney ATPases which was considerable and could have physiological effects.

DESAIAH and KOCH (1975) had very different results investigating the effect of toxaphene on ATPases of kidney, brain and gill of the catfish, Ictalurus punctatus. They reported lesser inhibitory effect on kidney than on brain ATPases. (O-I) Mg^{2+} ATPase of kidney showed a dose response to toxaphene. Toxaphene inhibited all 3 ATPases in brain to the extent of 50% with a pronounced effect on (O-I) Mg^{2+} ATPase. These results are of interest since toxaphene is a potent piscicide.

Toxaphene effects in vitro with cockroach ATPases displayed a different picture from that of the mouse. All ATPases were affected in a dose-response fashion. $Na^{+}K^{+}$ ATPase of both tissues tested exhibited inhibition by 10^{-4}M toxaphene slightly exceeding 50%. Mg^{2+} ATPase of the CNS was significantly inhibited. Results of in vitro administration of toxaphene on different tissues of the mouse, cockroach and catfish in the preceding three studies seem to indicate that ATPases differ in their response to toxaphene depending on the tissue and the animal. Most of the ATPases tested in our study show a significant dose response to toxaphene. However, the degree of inhibition, with the exception of $Na^{+}K^{+}$ ATPase of kidney, CNS and Malpighian tubules, was very low and might not have a physiological significance.

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