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Inhibition of Calcium ATPase Activity in Rat Brain and Muscle by Chlordecone

S. K. Mishra, M. Koury, and D. Desaiah

Neurology and Research Services, Veterans Administration Medical Center and Department of Neurology, The University of Mississippi Medical Center, Jackson, MS 39216 USA

Since the occupational exposure of factory workers to chlordecone (Kepone^{R}) in 1975, (ANONYMOUS 1975) interest in unraveling its toxic mechanism(s) has increased markedly. It has been reported that chlordecone produces typical DDT-like tremors in both humans and experimental animals (CANNON et al. 1978, LARSON et al. 1979). The production of severe tremors was thought to be due to the involvement of the central nervous system (COHN et al. 1976). A clear understanding of the etiology of the development of tremors in chlordecone poisoning is not understood. The involvement of either energy (ATP) depletion or synaptic dysfunction might result in toxic symptoms (DESAIAH et al. 1977 a, b, 1980; DESAIAH 1980). We have recently reported that Na^{+}-K^{+} ATPase in mouse brain synaptosomes was significantly decreased by chlordecone both *in vitro* and *in vivo* (DESAIAH et al. 1978, 1980). Other studies to be published elsewhere, also demonstrated that chlordecone inhibits uptake of neurotransmitters by rat brain synaptosomes.[1] The relationship between the inhibition of Na^{+}-K^{+} ATPase and decreased uptake of neurotransmitters (dopamine and norepinephrine) in synaptosomes by chlordecone may well suggest that the CNS is involved in the toxicity of chlordecone. However, other ATP-utilizing enzymes (e.g., Ca^{2+} ATPase) in brain synaptosomes and in muscle were not investigated. The role of Ca-ATPase in muscle is well extablished (HASSELBACH & MAKINOSE 1961, MARTONOSI & FERETOS 1964, MACLENNAN 1970). Any change in this enzyme activity may alter the Ca^{2+} pump activity and therefore result in the impairment of muscle contraction and relaxation processes (MARTONOSI 1968).

The purpose of the present investigations was to determine the effects of chlordecone on muscle and brain Ca^{2+} ATPase activity and to investigate its effect on ^{45}Ca binding to the muscle sarcoplasmic reticulum and brain synaptosomal preparations.

MATERIALS AND METHODS

Male Sprague-Dawley rats, about 200 g each, (Charles River Breeding Lab.) were used. Chlordecone (99% pure) was a gift from Allied Chemical Corp. ^{45}Ca ($CaCl_2$) was purchased from New England Nuclear Corp.

Rats (4-6 per group) were treated orally with various levels of chlordecone dissolved in corn oil so as to receive 0, 10 and 25

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mg/kg/day for 3 days and 50 mg/kg/day for 2 days. All the rats received 0.3 mL corn oil. Animals were killed 36 h after the last dose by decapitation and the brain and hind leg muscle tissues were rapidly removed. For in vitro experiments, normal rats were used as tissue source.

The whole brains were placed in ice-cold sucrose solution (0.32 M sucrose and 10 mM imidazole, pH 7.5) and synaptosomes were prepared using a slightly modified procedure of COTMAN & MATTHEWS (1971). Each brain was homogenized in 9 volumes of sucrose solution using a ground glass homogenizer. The homogenate was centrifuged at 750 x g for 10 min and the pellet was discarded. The supernatant was centrifuged at 17,000 X g for 10 min and the pellet was resuspended in sucrose solution and centrifuged again at 17,000 X g for 10 min. The pellet was resuspended in 10 mL sucrose solution and layered on a two-step discontinuous Ficoll-sucrose gradient, consisting of 13% (w/v) Ficoll in 0.32 M sucrose and 7.5% Ficoll (w/v) in 0.32 M sucrose. After centrifugation at 65,000 X g for 45 min, the synaptosomal fraction was obtained at the interface of the 7.5 - 13% Ficoll-sucrose layer. The synaptosome band was removed, diluted with 9 volumes of sucrose solution and centrifuged at 17,000 X g for 10 min. The synaptosomal pellet was resuspended in sucrose solution, divided into small aliquots and quick frozen in liquid nitrogen. The frozen samples were stored at - 85°C until used.

The leg muscles from control and treated rats were removed and placed in an ice-cold solution of 0.32 M sucrose and 10 mM histidine, pH 7.5. The muscle (2g) from each rat was teased apart and cleared of any nerves and connective tissue. The tissue was minced and homogenized in 9 volumes of sucrose solution using a polytron homogenizer for 2 min at highest speed while keeping the tissue of 0-4°C. The sarcoplasmic reticulum fractions were prepared essentially according to the method described by SAMAHA & GERGELY (1965). The homogenate was centrifuged at 900 X g for 10 min to remove nuclei and cell fragments. The supernatant was then centrifuged at 10,000 X g for 30 min to remove the mitochondria. The resulting supernatant was then centrifuged at 65,000 X g for 1 h and the pellet (sarcoplasmic reticulum) was resuspended in sucrose solution, divided into small aliquots, quick frozen in liquid nitrogen and stored at - 85°C until used.

ATPase activity in rat brain synaptosomes and muscle sarcoplasmic reticulum was measured essentially according to the coupled enzymatic method described by FRITZ & HAMRICK (1966) and as reported by DESAIAH & HO (1979). The reaction medium contained 5 mM ATP, 5 mM Mg^{2+}, 0.5 mM Ca^{2+}, 135 mM imidazole-HCl buffer (pH 7.5), 0.2 mM NADH, 0.5 mM phospho-enolpyruvate, approximately 9 units of pyruvate kinase and 21 units of lactic acid dehydrogenase. A 50 µL synaptosomal or sarcoplasmic reticulum sample with a protein content of 20 to 30 µg was added. Absorbance

[1]Desaiah, D.: Inhibition of ^{3}H-Catecholamine uptake by brain synaptosomes in Kepone and mirex pre-treated rats (MS Submitted to Biochem. Pharmacol.)

changes in reaction mixture were measured at 340 nm with temperature controlled at 37°C. The change at 340 nm over a period of 10 min was used in calculating the specific activity. Enzyme activities were expressed as micromoles of P_i/mg protein/hr. Protein was determined by the method of LOWRY et al. (1951) using bovine serum albumin as standard.

Total ATPase activity was measured with Ca^{2+} and Mg^{2+} present in the reaction mixture. The Mg^{2+} ATPase activity was measured in the presence of 5 mM EGTA, which is a specific inhibitor of Ca^{2+} ATPase. Ca^{2+} ATPase activity was calculated as the difference between the total and Mg^{2+} ATPase activities. For assessing the in vitro effect of chlordecone on the enzyme activities, various concentrations of chlordecone were added to the reaction mixture with protein sample present and incubated for 5 min before the reaction was initiated by the addition of ATP. For in vivo experiments, the tissue fractions prepared from chlordecone pretreated rats were used for determinations of ATPase activities.

The binding of ^{45}Ca to muscle sarcoplasmic reticulum and brain synaptosomes prepared from normal rats was determined using a slightly modified procedure described by SAMAHA & GERGELY (1965). The reaction mixture (1mL) used contained 8 mM Mg^{2+}, 6 mM ATP, 100 mM K^+, 50 mM Tris, pH 7.4 and 15 µM ^{45}Ca. About 30-50 µg of muscle sarcoplasmic reticulum or brain synaptosomal protein were used for each assay. The non-specific binding was obtained by incubating the reaction with 10 mM "cold" Ca^{2+} for 30 min before the addition of ^{45}Ca. Blanks contained no enzyme. The reaction mixture was incubated for 30 min more in the presence of ^{45}Ca and filtered using a 0.45 micron size millipore filters. By using two 12 place manifolds we could speed up the filtering process. The filters were removed, air dried, transferred to Aquasol and the radioactivity was counted in a liquid scintillation counter. Whenever chlordecone was present the reaction mixture with tissue fraction was preincubated for 5-30 min before the addition of ^{45}Ca.

RESULTS

Rats treated with chlordecone at all levels showed less weight gain than the controls. A maximum of 20% reduction was seen in the 50 mg/kg/day group within 3 days after treatment. The rats in the 25 and 50 mg/kg groups showed severe tremors characteristic of neurotoxicity. These results are in agreement with those reported in mice (DESAIAH et al. 1978).

In vitro response of Ca^{2+} (EGTA-sensitive) and Mg^{2+} (EGTA-insensitive) ATPases in muscle to different concentrations of chlordecone are presented in Table 1. Both Ca^{2+} and Mg^{2+} ATPase activities were inhibited by chlordecone in a dose-dependent manner with approximate ID_{50} values of <5 and <10 µM respectively. Ca^{2+} ATPase was more sensitive to chlordecone than Mg^{2+} ATPase at any concentration tested in muscle preparations. The sensitivity of brain synaptosomal Ca^{2+} and Mg^{2+} ATPase activities to chlordecone were also determined and the data obtained are presented in Table 2.

TABLE 1. Effect of chlordecone on Ca^{2+} and Mg^{2+} ATPase activities in rat muscle sarcoplasmic reticulum in vitro

Concentration	Specific Activity ± S.D.[a]	
(μM)	Ca^{2+} ATPase	Mg^{2+} ATPase
0	4.7 ± 1.9	2.9 ± 1.2
1	3.7 ± 0.7	2.8 ± 1.0
5	1.2 ± 0.4[b]	1.4 ± 0.6
10	1.0 ± 0.2[b]	1.0 ± 0.4[b]
20	0.5 ± 0.1[b]	0.9 ± 0.2[b]

[a] Specific Activity is expressed as μ moles Pi/mg protein/h. Each value represents the means of 3 or more different preparations and each preparation was assayed in triplicate.
[b] Significantly different from control ($P<0.05$).

As in muscle, the brain enzymes also were inhibited by chlordecone in a dose-dependent manner, reaching a maximum inhibition of 75% at the highest concentration tested. The approximate ID_{50} values were >5 and 5μM for Ca^{2+} and Mg^{2+} ATPase respectively. In contrast to muscle the two brain ATPases are more or less equally sensitive to chlordecone in vitro.

TABLE 2. Effect of chlordecone on Ca^{2+} and Mg^{2+} ATPase activities in rat brain synaptosomes in vitro

Concentration	Specific Activity ± S.D.[a]	
(μM)	Ca^{2+} ATPase	Mg^{2+} ATPase
0	3.7 ± 1.0	16.1 ± 2.0
1	2.1 ± 0.5	13.8 ± 0.9[b]
5	2.0 ± 0.6	7.8 ± 1.3[c]
10	0.8 ± 0.3[b]	7.3 ± 1.8[c]
20	0.9 ± 0.0[b]	4.1 ± 1.1[c]

[a] Specific Activity is expressed as μ moles Pi/mg protein/h. Each value represents the means of 3 or more different preparations and each preparation was assayed in triplicate.
[b] Significantly different from control ($P<0.05$).
[c] Significantly different from control ($P<0.001$).

The Ca^{2+} (EGTA-sensitive) and Mg^{2+} (EGTA-insensitive) ATPase activities of muscle sarcoplasmic reticulum and brain synaptosomes prepared from chlordecone treated and control rats are presented

in Tables 3 and 4. The Ca^{2+} ATPase activity in both muscle and brain were unaffected by chlordecone treatment. The Mg^{2+} ATPase activity was also unaffected in brain, however, the muscle Mg^{2+} ATPase activity in the rats receiving highest doses of chlordecone was increased 2-3 fold. The increase was significant and dose-dependent (Table 3).

TABLE 3. Effect of *in vivo* pretreatment of chlordecone on Ca^{2+} and Mg^{2+} ATPase activities in rat muscle sarcoplasmic reticulum

Treatment Dose (mg/kg/day)	No. Days Treated	Specific Activity ± S.D.[a] Ca^{2+} ATPase	Specific Activity ± S.D.[a] Mg^{2+} ATPase
0 (corn oil)	3	3.9 ± 0.7	2.5 ± 0.7
10	3	3.1 ± 0.0	3.2 ± 0.7
25	3	5.5 ± 2.6	5.7 ± 1.2[b]
50	2	3.8 ± 0.7	8.0 ± 2.4[b]

[a] Specific Activity is expressed as μ moles Pi/mg protein/h. Each value represents the means of 3 or more different preparations and each preparation was assayed in triplicate.
[b] Significantly different from control ($P<0.05$).

TABLE 4. Effect of *in vivo* pretreatment of chlordecone on Ca^{2+} and Mg^{2+} ATPase activities in rat brain synaptosomes

Treatment Dose (mg/kg/day)	No. Days Treated	Specific Activity ± S.D.[a] Ca^{2+} ATPase	Specific Activity ± S.D.[a] Mg^{2+} ATPase
0 (corn oil)	3	2.0 ± 1.4	12.4 ± 2.0
10	3	1.7 ± 0.3	13.1 ± 2.0
25	3	3.2 ± 1.2	10.5 ± 0.6
50	2	2.9 ± 1.1	11.1 ± 1.3

[a] Specific Activity is expressed as μ moles Pi/mg protein/h. Each value represents the means of 3 or more different preparations and each preparation was assayed in triplicate.

The binding of ^{45}Ca to muscle sarcoplasmic reticulum and brain synaptosomal preparations was determined *in vitro* in the absence and presence of chlordecone (100 and 200 μM) and the data are presented in Table 5. When the normal tissue fractions were preincubated with chlordecone for 5-30 min, the binding of ^{45}Ca

was uneffected. Since chlordecone had no effect *in vitro* on ^{45}Ca binding further work with chlordecone pretreated fractions was not under taken.

TABLE 5. Effect of chlordecone on ^{45} Calcium binding to rat brain synaptosomes and muscle sarcoplasmic reticulum *in vitro*

Chlordecone	CPM/mg protein/10 min	
(μM)	Synaptosomes	Sarc. Ret.
0	188 ± 10	677 ± 26
100	177 ± 7	703 ± 19
200	199 ± 16	662 ± 32

DISCUSSION

Chlordecone has been shown to interfere with energy metabolism in fish (DESAIAH & KOCH 1975) rat (DESAIAH et al. 1977 a,b, 1980; DESAIAH 1980) and in isolated P 388 D_1 cells (CARMINES et al. 1979). REITER et al. (1977) reported that chlordecone induces hyperactivity in rats similar to the tremors produced in humans exposed to chlordecone in a manufacturing facility (MARTINEZ et al. 1977). CARMINES et al. (1979) have suggested that the observed neurotoxic symptoms *in vivo* may be related to the impaired energy metabolism and cellular calcium distribution. END et al. (In press, referred to by CARMINES et al. 1979) have demonstrated that chlordecone blocks the uptake of calcium in the rat brain mitochondria and synaptosomes which in turn results in the release of neurotransmitters at synaptic junction. However, these authors have not determined the synaptosomal Ca^{2+} ATPase sensitivity to chlordecone. Our present data demonstrate that chlordecone inhibits Ca^{2+} ATPase activity in rat brain synaptosomes *in vitro*. A concentration dependent inhibition of Ca^{2+} ATPase was observed with an approximate ID_{50} of 5μM chlordecone. On the other hand, we failed to see any change in Ca^{2+} ATPase in the rats pretreated with chlordecone at 10, 25 and 50 mg/kg/day for 3 days. The Mg^{2+} ATPase in liver mitochondria of rats treated with similar doses resulted in a 40% decrease as compared to controls receiving vehicle only (DESAIAH et al. 1977 b). Additionally in our laboratory, chlordecone (up to 200 μM) had no effect on ^{45}Ca binding to brain synaptosomes.

RUBIN (1974) has reported that calcium plays a significant role in muscle excitation and contraction coupling and neurotransmitter release. Sarcoplasmic reticulum is known to accumulate calcium and a biochemical manifestation for the Ca^{2+} pump is the Ca^{2+} ATPase. To evaluate the effects of chlordecone on Ca^{2+} pump activity we determined the sensitivity of Ca^{2+} ATPase to chlordecone both *in vitro* and *in vivo*. The data presented in this paper show that chlordecone inhibits Ca^{2+} ATPase *in vitro* but not *in vivo*.

The apparent descrepancy of the responses of Ca^{2+} ATPase and calcium binding in brain and muscle to chlordecone in vitro and in vivo might be due to that the treatment time (3 days) might have been too short for chlordecone to alter the Ca^{2+} pump activity in vivo. Nevertheless, if chlordecone indeed has no effect on Ca^{2+} pump activity in vivo, it is possible that its main effect may be via the mitochondrial energy metabolism as evidenced by our previous studies (DESAIAH et al. 1977 a, b, 1980; DESAIAH 1980).

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