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# DDT INHIBITION OF Ca-Mg ATPase FROM PERIPHERAL NERVES AND MUSCLES OF LOBSTER, HOMARUS AMERICANUS

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SUMMARY: Sensitivity of Ca-Mg ATPase from axonic plasma membrane (APM) and sarcoplasmic reticulum (SR) of lobster, Homarus americanus, to DDT was studied. The Ca-Mg ATPase found in SR with the high $Ca^{2+}$ affinity is sensitive to DDT while the portion of ATPase related to the low $Ca^{2+}$ affinity site is not inhibited by DDT. Also, DDT is more inhibitory against the Ca-Mg ATPase prepared from APM than the one obtained from SR. The relationship between inhibition of the Ca-Mg ATPase by DDT in the axonic nerve membrane and in vivo poisoning symptoms of the nervous system is discussed.

DDT is known to disrupt the functions of the axonic membranes, including those in the peripheral nervous system. It has been shown that several ATPases present in the nervous system are sensitive to DDT inhibition (1-5). Since DDT has been found to inhibit Ca-ATPase of nerve membrane (6-8), as well as Ca-Mg ATPase from sarcoplasmic reticulum (9,10), a question must be raised as to which of these calcium-stimulated ATPases is more sensitive, and how their inhibition is related to toxic actions of DDT. In this project we have made an attempt to answer this particular question and now report the result.

## MATERIALS AND METHODS

### Preparation of Enzyme

Sarcoplasmic reticulum (SR) and axonic plasma membrane (APM) from lobster (Homarus americanus) tail muscles and peripheral nerves, respectively, were used as the enzyme source for ATPase studies. The details of the enzyme preparations were as follows:

(a) SR was prepared according to the methods of Huddart et al. and Price (9,10). About 3-5 g of lobster tail muscles were homogenized at $0^{\circ}C$ for 3-5 minutes using a teflon-glass Potter-Elvehjem homogenizer in 1 mM imidazol buffer (pH 7.0) in the co-presence of 100 mM potassium chloride (KCl), and 1 mM dithiothreitol (DTT). To

Abbreviations used: DDT, 1,1,1-trichloro-2,2-bis-(p-chlorophenyl)-ethane; SR, sarcoplasmic reticulum; APM, axonic plasma membrane; DTT, dithiothreitol; BSA, bovine serum albumin; Pi, inorganic phosphate; EGTA, ethyleneglycol-bis-(ß-amino-ethyl ether) N,N'-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

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remove cell debris, the homogenate was first centrifuged in International Refrigerated Centrifuge (Model PR-2) at 3000 g for 10 min. The supernate was filtered through glass-wool to eliminate excess of freely floating lipids from the preparation. The mitochondria were then sedimented by centrifugation at 8000 g for 30 min. The supernate from this spin was centrifuged at 28,000 g for 60 min using a Beckman Model L-2 Ultracentrifuge to obtain the SR pellet.

SR pellet thus prepared was resuspended in the imidazol buffer (pH 7.2), divided into smaller portions, and either quick frozen and stored at-20°C, or held at 0°C for 1-2 days until required for the assays.

(b) Lobster nerve APM was prepared as described earlier (6,7). Protein concentration of the enzyme was determined by the colorimetric method of Lowry et al. (11), using a bovine serum albumin (BSA) as the standard.

ATPase Assay Procedure

For the assay of Ca-Mg ATPase, a calcium - EGTA equilibrium system was employed (Portzehl et al., 12). The inorganic phosphate (Pi) formed as a result of $\gamma$-$^{32}$P ATP hydrolysis was measured by radiometric charcoal method as described by Yamaguchi et al. (13), and used as the measure of ATPase activity.

The total volume of the assay mixture was 0.9 ml buffer plus 0.1 ml of enzyme preparation. The ionic composition of the assay mixture was simliar to as given by Robinson (14) and Yamaguchi et al. (13). This consisted of 30 mM imidazol buffer (pH 7.2) plus 2 mM ATP (tris-salt preadjusted with $\gamma$-$^{32}$P ATP), 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM ouabain, 0.5 mM EGTA and 0.45 mM $Ca^{2+}$ (equivalent to 5 x $10^{-6}$M free $Ca^{2+}$ as calculated from Portzehl et al. (12), all expressed as final concentrations. The Ca-Mg ATPase is described as the ATPase activity obtained in the above reaction mixture minus basal Mg-ATPase activity obtained in identical reaction mixture and assay conditions, except for the absence of $Ca^{2+}$.

All chemicals used were of analytical reagent grade (AR) or of highest grade commercially available. Ouabain, imidazol, EGTA, EDTA, DTT, BSA, and cold ATP (tris-salt) were obtained from "Sigma," St. Louis, MO. $\gamma$-$^{32}$P ATP (specific activity 20-35 Ci/mmol) was obtained from New England Nuclear, Boston, MA.

## RESULTS

Effect of Calcium Ions

Fig. 1 shows the effect of $Ca^{2+}$ on ATPase activity of SR of lobster tail muscles. It is evident that the enzyme activity of SR is stimulated by a wide range ($\mu$m to mM) of $Ca^{2+}$ concentrations (Fig. 1A). Activation of the enzyme at very low concentrations of $Ca^{2+}$ (such as 5 x $10^{-6}$M or less) in the presence of 3 x $10^{-3}$M $Mg^{2+}$ indicates the presence of Ca-Mg ATPase in the muscle. Under the assay condition SR had a mean basal Mg-ATPase activity of 3.83 $\mu$mol Pi released/mg/10 min. When 5 x $10^{-6}$M of free $Ca^{2+}$ was used as a mixture of 0.5 mM EGTA plus 4.5 x $10^{-4}$M $Ca^{2+}$ in the above basal Mg-ATPase medium, the mean total ATPase increased to 6.41 $\mu$mol Pi liberated/mg/10 min., giving a maximum activation of about 167%. Greater activations of ATPase by $\mu$M concentrations of $Ca^{2+}$ are also reported with synaptic preparations from rat (147%), sheep (200%), and pig (269%) brains (15). When the basal

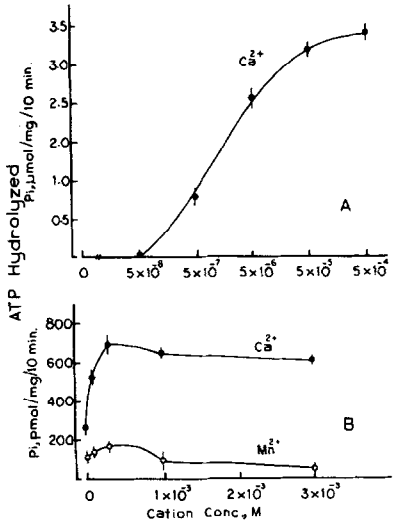


Fig. 1. Effect of cation concentration on ATPase activity of sarcoplasmic reticulum (SR) from lobster tail muscles at 30°C. (A) shows the effect of $Ca^{2+}$ on Ca-Mg ATPase activation at 2 mM ATP concentration while (B) shows the effect of $Ca^{2+}$ and $Mn^{2+}$ on muscle ATPase when tested under ecto Ca-ATPase conditions at low ATP concentration (8 x $10^{-6}$ M) [also see (6,7)].

Mg-ATPase activity was subtracted from the one obtained with $Mg^{2+}$ + $Ca^{2+}$, the net specific activity of Ca-Mg ATPase was found to be 2.58 µ mol Pi liberated/mg/10 min. (Fig. 1A).

The effect of $Ca^{2+}$ on activation of the SR ATPase under the assay conditions of Ca-ATPase [see Matsumura and Ghiasuddin (6)] is shown in Fig. 1B. $Ca^{2+}$ in the absence of $Mg^{2+}$ stimulates the ATPase from SR and produced maximum activation at an optimum concentration of 0.3 mM, as observed earlier with nerve membrane fraction (7). The total effect of $Ca^{2+}$ observed in SR was, however, much less (i.e., 692 pmol/mg/10 min.) than that observed earlier using lobster nerve preparations (7,8).

Ecto Ca-ATPase is also a Ca-ATPase stimulated by several divalent cations (16), and in the case of the lobster nerve membrane is best activated by $Mn^{2+}$ rather than $Ca^{2+}$ (17). Therefore, in another set of experiments, $Ca^{2+}$ was replaced with $Mn^{2+}$. Results obtained are co-plotted with the data on $Ca^{2+}$ effect for close comparison. As shown in Fig. 1B, $Mn^{2+}$ is very poor in activation of the ATPase from SR, suggesting, thereby, that it could not be the same enzyme as ecto Ca-ATPase of the nervous system.

Table 1

Sensitivity of lobster nerve membrane and muscle Ca-Mg ATPases to DDT at 30°C

| DDT Concentration (M)[b] | % Inhibition[a] | | |
|---|---|---|---|
| | Ca-Mg ATPase[c] | | Ca-ATPase[c] |
| | Muscle-SR | Axonic Membrane | Muscle-SR |
| $10^{-5}$ | 49.1 ± 3.4 | 95.6 ± 1.9 | 0 |
| 10-6 | 36.7 ± 2.0 | 63.8 ± 3.7 | 0 |
| $10^{-7}$ | 31.2 ± 4.1 | 38.4 ± 2.8 | 0 |
| $10^{-8}$ | 24.8 ± 1.8 | 21.6 ± 2.2 | 0 |
| $10^{-9}$ | 2.2 ± 0.8 | 3.5 ± 2.0 | 0 |

[a]The protein concentration was 0.088 mg/assay tube for muscle sarcoplasmic reticulum (SR), and 0.032 mg/assay tube for axonic membrane preparation. Results are averages of at least three independent experiments with two replicates in each set and expressed as mean % inhibition with standard errors (± S.E.).

[b]DDT was added in 10 µl ethanol while an equivalent amount of ethanol was added to control.

[c]The specific activity of Ca-Mg ATPase from muscle-SR and nerve axonic plasma membrane using 2 mM ATP as a final concentration was 2.58 µ mol/mg/10 min. and 0.82 µ mol/mg/10 min., respectively, while the specific activity of Ca-ATPase from lobster tail muscle-SR was 692 pmol/mg/10 min., at a final substrate concentration of $8 \times 10^{-8}$ M ATP [see (6,7)].

Effect of DDT on Ca-Mg ATPase

Results on the effect of DDT on $Ca^{2+}$ stimulated ATPase from SR, and Ca-Mg ATPase from SR and APM are summarized in Table 1. DDT inhibited the Ca-Mg ATPase obtained from both sources. However, the Ca-Mg ATPase from the nerve preparation was relatively more sensitive than the one obtained from the muscle. In another set of experiments the inhibitory effect of DDT on $Ca^{2+}$ stimulated ATPase of SR was studied. Unlike the case with Ca-Mg ATPase, DDT failed to inhibit this enzyme at all the concentrations tested (Table 1).

DDT effect on Ca-Mg ATPase and $Ca^{2+}$ stimulated ATPase from SR at different temperatures is given in Fig. 2A and Fig. 2B. Ca-Mg ATPase was inhibited by DDT at all the temperatures tested (Fig. 2A), but once again Ca-stimulated ATPase from the

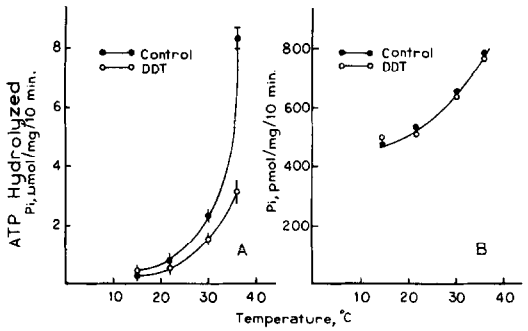


Fig. 2. Effect of temperature on the sensitivity of $Ca^{2+}$ stimulated ATPase from the muscle SR to DDT (tested at $10^{-5}$M final concentration). (A) shows inhibition of Ca-Mg ATPase under the identical assay conditions as in Fig. 1 at the 5 x $10^{-6}$M level of free $Ca^{2+}$, while (B) shows the effect of DDT under the conditions used for ecto Ca-ATPase, as in Fig. 1, using 0.3 mM $Ca^{2+}$ as optimum.

SR, when tested at different temperatures, was found to be totally insensitive to DDT (Fig. 2B). A closer examination of the data shows a positive correlation between the % inhibition of Ca-Mg ATPase and the incubation temperature (Fig. 2A). This observation is in contrast to the characteristic negative correlation of toxic action of DDT and the test temperatures, as observed with Ca-ATPase of lobster nerve preparations (7,8).

## DISCUSSION

Characteristics of Ca-stimulated ATPases in terms of localization in tissues (i.e., muscle and nervous system), and their sensitivities to added cations are listed in Table 2. Under the experimental conditions two $Ca^{2+}$ stimulated systems are suspected to be present in the axon rich plasma membrane preparation. One is ecto Ca-ATPase described by Trams et al. (16), and the portion of Ca-Mg ATPase due to the low $Ca^{2+}$ affinity site (18). However, in SR it was found that $Mn^{2+}$ was found to be a poor substitute of $Ca^{2+}$, indicating that this Ca-stimulated ATPase activity is not likely due to the ecto ATPase (see also 17). Instead it seems more logical to conclude that the Ca-ATPase activity found in SR is most likely to be the portion of Ca-Mg ATPase activity related to the low $Ca^{2+}$ affinity site as pointed out by Ikemoto (18).

In our view the most significant findings of this work are that (a) the Ca-Mg ATPases are generally sensitive to DDT inhibition, (b) the Ca-Mg ATPase from the

Table 2

Characteristics of four different types of ATPases that are stimulated by $Ca^{2+}$

| Enzyme | Main Site of Localization | Characteristics |
|---|---|---|
| Ca-Mg ATPase | Muscle SR[a]<br>Nerve synaptic ER[a] | Requires both $Mg^{2+}$ (mM range) and $Ca^{2+}$ (μM range for high affinity site and mM range for low affinity site). |
| ecto Ca-ATPase | Nerve plasma membrane (outer surface) | $Mn^{2+}$ (mM) stimulates the activity followed by $Ca^{2+}$ and $Mg^{2+}$. |
| Ca-ATPase | Nerve plasma membrane | Activated by $Ca^{2+}$ (in mM range). Active at low ATP concentration, sensitive to DDT. |
| Myosin-type ATPase | Various parts, including the inner surface of plasma and synaptic membranes | Activated by $Mg^{2+}$ or $Ca^{2+}$ (mM). Dissolves at a high $K^{+}$ (600 mM range). |

[a]SF: sarcoplasmic reticulum, ER: endoplasmic reticulum

nerve axonic membrane is more sensitive to DDT than the one from the muscle SR, and (c) the DDT sensitive site of Ca-Mg ATPase is not the low $Ca^{2+}$ affinity site.

As for the question of the relative importance of the nerve Ca-Mg ATPase in comparison with the nerve Ca-ATPase (6,7) in the DDT poisoning processes of the nervous system, one could point out from the difference in the apparent $I_{50}$ ($5 \times 10^{-7}$ M for the former and $2 \times 10^{-9}$ for the latter) that the latter enzyme is more likely to be important in this regard. However, the question of actual roles of these enzyme systems in vivo in relation to poisoning with any insecticide is not easy to answer, as other factors such as the localization, the quantity of enzymes and specific activities, and the relative importance of their roles in different parts of the nervous system, etc. are needed to be considered. Nevertheless, to answer such a question the first

requirement is to establish the sensitivity of the proposed target enzymes. In this regard, the finding of this work helps to clearly identify the presence of another sensitive enzyme system which could be in the future linked to the in vivo poisoning symptoms, and thereby explain some aspect of the phenomenon of DDT poisoning.

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