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# Separate Effects of Mercurial Compounds on the Ionophoric and Hydrolytic Functions of the $(Ca^{++}+Mg^{++})$-ATPase of Sarcoplasmic Reticulum

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*Summary.* We have shown that a $Ca^{++}$-ionophore activity is present in the $(Ca^{++}+Mg^{++})$-ATPase of rabbit skeletal muscle sarcoplasmic reticulum (A.E. Shamoo & D.H. MacLennan, 1974. *Proc. Nat. Acad. Sci. USA* **71**:3522). Methylmercuric chloride inhibited the $(Ca^{++}+Mg^{++})$-ATPase and $Ca^{++}$ transport, but had no effect on the activity of the $Ca^{++}$ ionophore. Mercuric chloride inhibited ATPase, transport and ionophore activity. The ATPase and transport functions were more sensitive to methylmercuric chloride than to mercuric chloride. The two functions were inhibited concomitantly by methylmercuric chloride but slightly lower concentrations of mercuric chloride were required to inhibit $Ca^{++}$ transport than were required to inhibit ATPase. Methylmercuric chloride and mercuric chloride probably inhibited ATPase and $Ca^{++}$ transport by blocking essential -SH groups. However, it appears that there are no essential -SH groups in the $Ca^{++}$ ionophore and that mercuric chloride inhibited the $Ca^{++}$ ionophore activity by competition with $Ca^{++}$ for the ionophoric site. Blockage of $Ca^{++}$ transport by mercuric chloride probably occurs both at sites of essential -SH groups and at sites of ionophoric activity. These data suggest the separate identity of the sites of ATP hydrolysis and of $Ca^{++}$ ionophoric activity.

We have recently published several reports on the existence and isolation of ion-transport mediators (ionophores) from a variety of tissues (Shamoo & Albers, 1973; Blumenthal & Shamoo, 1974; Shamoo, 1974; Shamoo & Myers, 1974; Shamoo, Myers, Blumenthal & Albers, 1974), including the isolation of a $Ca^{++}$-dependent and selective ionophore from the $(Ca^{++}+Mg^{++})$-ATPase of sarcoplasmic reticulum of rabbit skeletal muscle (Shamoo & MacLennan, 1974). This discovery makes it possible to study separately the ionophoric site (ion-mediating site) and the energetic site (ATP hydrolytic site) of the $(Ca^{++}+Mg^{++})$-ATPase enzyme. In addition, it is possible to observe effects on these sites in sarcoplasmic reticulum vesicles, where $Ca^{++}$-uptake and $Ca^{++}$-binding can be measured directly.

Observations of the action of mercurial compounds on ATP hydrolysis, $Ca^{++}$ transport and $Ca^{++}$ ionophoric activity can help to elucidate the basic mechanism of the active transport of $Ca^{++}$ and, moreover, can provide operational parameters for attempts to reverse the toxic effects of these agents. In a preliminary report we have shown that the $Ca^{++}$-ionophore was inhibited with $HgCl_2$ but not with $CH_3HgCl$ (Shamoo, MacLennan & Eldefrawi, 1975).

In the present report we present evidence for differential effects of methylmercuric chloride and mercuric chloride on the $Ca^{++}$-ionophore and on the $Ca^{++}$ transport-ATPase. The data suggest that the sites of $Ca^{++}$-ionophore activity and of ATP hydrolysis are separate.

## Materials and Methods

### *Isolation of Proteins from Sarcoplasmic Reticulum*

Sarcoplasmic reticulum and the $(Ca^{++} + Mg^{++})$-dependent ATPase were isolated from rabbit skeletal muscle by the method of MacLennan (1970). The enzyme was dissolved in 1% cholate, passed through a G200 Sephadex column in 1% cholate to remove phospholipid, and was then succinylated to make it soluble in the absence of cholate (Shamoo & MacLennan, 1974). Succinic anhydride (5 mg) was added slowly to 1 mg of protein while the pH was maintained at pH 7–8 with NaOH. After completion of the reaction the protein was dialyzed against several changes of 10 mM Tris-HCl, pH 8.0 to remove cholate. This soluble preparation appeared as a single species, of molecular weight 100,000 on SDS-polyacrylamide gels. This soluble preparation was the material tested as the $Ca^{++}$-ionophore.

### *Sarcoplasmic Reticulum Assays*

*Preincubation.* Various dosages of methylmercuric chloride or mercuric chloride were tested by incubating the protein [whether sarcoplasmic reticulum or $(Ca^{++} + Mg^{++})$-ATPase] with methylmercuric chloride or mercuric chloride at the indicated preincubation dosage (first dosage) followed by the introduction of the enzymic reaction mixture which led to the indicated second dosage. Frozen sarcoplasmic reticulum was thawed and diluted to 6 mg/ml with 0.25 M sucrose, 10 mM Tris, 1 mM histidine, pH 8.0. Two tenths ml was incubated for 5 min at 37 °C in the presence of the indicated concentrations of $HgCl_2$ or $CH_3HgCl$ in a total volume of 0.3 ml. The samples were then placed on ice and assayed for ATPase and $Ca^{++}$-transport. Preincubation in the absence of ATP and divalent metals was essential to inhibit -SH groups which might be protected by these reagents.

### *Calcium Transport and ATPase Assay*

$Ca^{++}$ transport was measured according to the method of Sommer and Hasselbach (1967). The reaction mixture contained, in 3 ml, 20 mM histidine, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM $Ca^{45}Cl_2$, 5 mM K oxlate, 5 mM ATP and 600 μg protein.

After 1, 3 and 5 min at 24 °C, 0.5 ml was filtered through a 0.3 μm millipore filter. The filter was washed with 5 ml of 0.15 M NaCl and examined in a scintillation spectrometer. For the simultaneous measurement of ATPase activity 1 ml of the reaction mixture was acidified with silicotungstate and inorganic orthophosphate was determined by method D as described by Lindberg and Ernster (1956). Protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951).

### *Conductance Measurement*

A Keithley Electrometer Model No. 616 was used either in series (as an ammeter) or in parallel (as a voltmeter) with the black lipid membrane (Shamoo & MacLennan, 1974). The electrometer output was connected simultaneously to a two-channel recorder. The voltage source was a function generator (IE F52A). Materials to be assayed were normally placed in both compartments separated by the bilayer. The bilayer-forming solution was oxidized cholesterol prepared according to the method of Tien, Carbone and Dawidowicz (1966). The membrane was formed after the addition of the ionophoric material into the designated bathing solution. The membrane diameter was 1 mm and the solution volume of the Teflon cup and the outside compartment was 5 ml. The conductance ($G$) was expressed in mho for the area of the membrane (1 mm diameter).

## Results

The effects of methylmercuric chloride and mercuric chloride were studied separately on three parameters related to sarcoplasmic reticulum: $Ca^{++}$-transport in sarcoplasmic reticulum, $(Ca^{++}+Mg^{++})$-ATPase activity in sarcoplasmic reticulum and in the $(Ca^{++}+Mg^{++})$-ATPase enzyme, and $Ca^{++}$-dependent and selective ionophoric activity in the succinylated ATPase protein. The amount of protein used in all of these experiments was constant. Therefore the concentrations indicated are directly proportional to number of moles of mercurial compounds per mg protein. If the amount of protein varies the free mercurial concentration would vary accordingly. Therefore, the number of moles of mercurials per mg protein is the parameter where percent inhibition can be compared.

### *Methyl Mercuric Chloride*

Fig. 1 shows the effects of various doses of methylmercuric chloride on $Ca^{++}$-transport and $(Ca^{++}+Mg^{++})$-ATPase in sarcoplasmic reticulum, determined simultaneously on the same sample. Methylmercuric chloride inhibited 50% of both parameters at $5\times10^{-5}$ M (1st dosage) and over 90% of both parameters at $2\times10^{-4}$ M (1st dosage). The two dose-effect curves ran closely in parallel. This result would be expected if methyl-

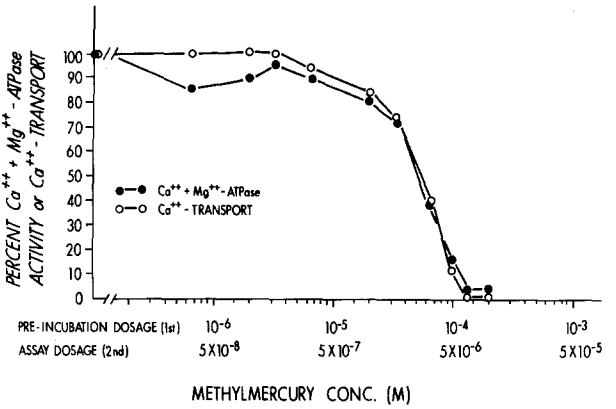


Fig. 1. Effect of methylmercury chloride on $(Ca^{++} + Mg^{++})$-ATPase and $Ca^{++}$ transport activities. *See* Materials and Methods for details concerning the conditions for preincubation and assay

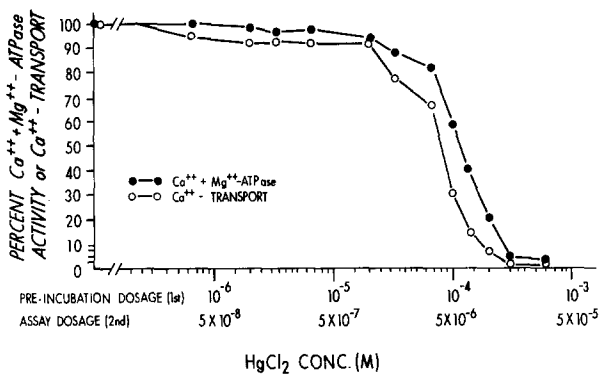


Fig. 2. Effect of mercuric chloride on $(Ca^{++} + Mg^{++})$-ATPase and $Ca^{++}$ transport activities. *See* Materials and Methods for detail

mercuric chloride acted by inhibiting a single rate-limiting step in both the ATPase activity and the active transport. This is consistent with the theory, that the energy source for $Ca^{++}$-transport comes from the hydrolysis of ATP by the $(Ca^{++} + Mg^{++})$-ATPase. The effects of various doses of methylmercuric chloride on $Ca^{++}$-transport and $(Ca^{++} + Mg^{++})$-ATPase were determined in four separate experiments. The dose-response curves (not shown) were closely similar to the two curves in Fig. 1.

## *Mercuric Chloride*

Fig. 2 shows that mercuric chloride also produced parallel dose-effect curves in inhibiting calcium transport and $(Ca^{++} + Mg^{++})$-ATPase. Mer-

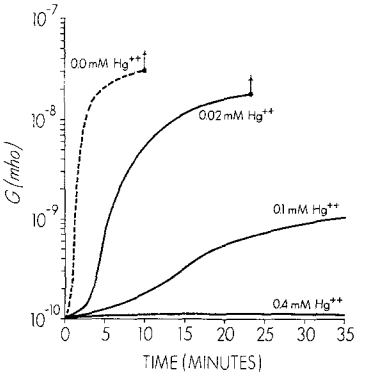


Fig. 3. Effect of mercuric chloride on the time-response of black lipid bilayer membrane conductance in the presence of $5 \times 10^{-4}$ mg/ml $Ca^{++}$-ionophore [solubilized $(Ca^{++}+Mg^{++})$-ATPase], 3 mM $CaCl_2$ and 5 mM histidine, pH 7.4. The conductance was continuously monitored under constant 50 mV across the bilayer. Similar data were obtained using the same bath and increasing the $HgCl_2$ concentration or with independent experiments with various doses of $HgCl_2$

curic chloride inhibited 50% of both parameters at approximately $10^{-4}$ M (1st dosage) ($8 \times 10^{-5}$ M for transport and $1.1 \times 10^{-4}$ M for ATPase), and over 90% of both parameters at $3 \times 10^{-4}$ M (1st dosage). Therefore, the inhibitory action of mercuric chloride is similar to that of methylmercuric chloride. The site of action is probably at an essential sulfhydryl group bound by the mercuric ion. Four more experiments on the effect of $HgCl_2$ on $(Ca^{++}+Mg^{++})$-ATPase activity and $Ca^{++}$-transport were carried out separately. The inhibitory action of $HgCl_2$ was similar to that shown in Fig. 2.

### *Effect of Mercurial Compounds on the $Ca^{++}$-Ionophore*

The effect of various doses of $HgCl_2$ on the conductance of a black lipid membrane is shown in Fig. 3. The concentration of $CaCl_2$ was 3 mM. The primary conducting ion in this system was calcium (Shamoo & MacLennan, 1974). When solubilized $(Ca^{++}+Mg^{++})$-ATPase (the $Ca^{++}$-ionophore) was added, the increase in conductance was completely blocked by 0.4 mM $HgCl_2$.

Methylmercuric chloride up to a maximum concentration of $10^{-3}$ M, had no effect on the $Ca^{++}$-ionophore activity. In the presence of $Ca^{++}$, the conductance of the black lipid membrane increased in response to the $Ca^{++}$-ionophore obtained from the $(Ca^{++}+Mg^{++})$-ATPase, and

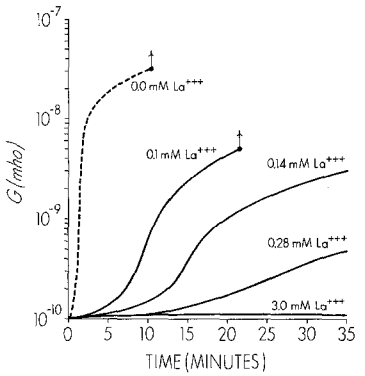


Fig. 4. Effect of lanthanum chloride on the time response of bilayer conductance in the presence of $5 \times 10^{-4}$ mg/ml $Ca^{++}$-ionophore, 5 mM histidine, pH 7.4, and 3 mM $CaCl_2$. Other conditions are similar to those described for Fig. 3

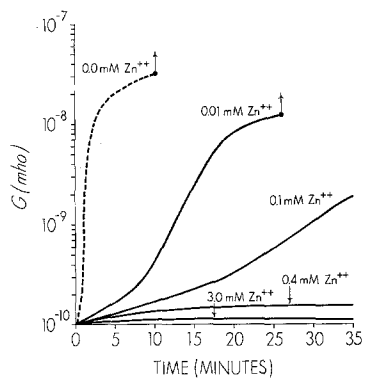


Fig. 5. Effect of zinc chloride on the time response of bilayer conductance in the presence of $5 \times 10^{-4}$ mg/ml $Ca^{++}$-ionophore, 3 mM $CaCl_2$, and 5 mM histidine, pH 7.4

the increase was the same with or without methylmercuric chloride. This indicates that the $Ca^{++}$-ionophore has no sulfhydryl groups essential for its activity.

The finding, that $HgCl_2$ blocked ionophoric activity completely while methylmercury, also a sulfhydryl group blocker, had no effect, indicated that the site of action of $HgCl_2$ was not a sulfhydryl group. Rather, the mechanism of inhibitory action of the divalent $Hg^{++}$ ion may be ascribed to a replacement of $Ca^{++}$ at the $Ca^{++}$ ionophoric site. If this hypothesis is true, the same kind of inhibition should be produced by other divalent or trivalent cations to the extent that they resemble $Ca^{++}$. This was confirmed for $La^{3+}$ and $Zn^{++}$ at doses closely similar to the inhibitory doses of $Hg^{++}$. $Mn^{++}$ also produced a parallel dose-effect curve in inhibiting the $Ca^{++}$-ionophore, but only at significantly higher doses (Figs. 4, 5, and 6).

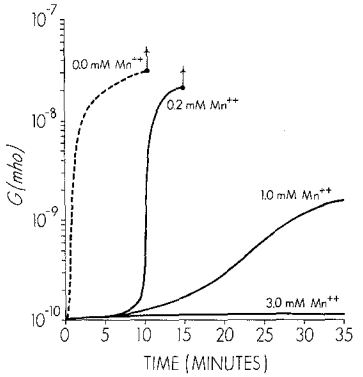


Fig. 6. Effect of manganese chloride on the time response of bilayer conductance in the presence of $5 \times 10^{-4}$ mg/ml $Ca^{++}$-ionophore, 3 mM $CaCl_2$ and 5 mM histidine, pH 7.4

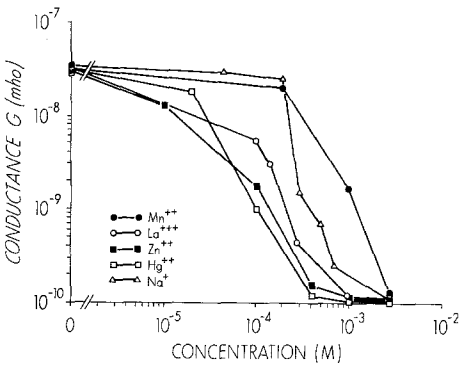


Fig. 7. Conductance versus cation concentrations. The maximum black lipid membrane conductance was taken from Figs. 4, 5, 6 and 8 and plotted against the various concentrations of the inhibiting cation

Fig. 7 summarizes the data in Figs. 4, 5, 6 and the coming Fig. 8. The Figure is a log-log plot of the black lipid membrane conductance in the presence of 3 mM $CaCl_2$ versus concentration of the indicated divalent cations shown in previous Figures. The conductance data were taken arbitrarily at their maximum value. It is clear from the Figure that $La^{3+}$, $Zn^{++}$ and $Hg^{++}$ inhibit the $Ca^{++}$ conductance with similar effective doses and that $Mn^{++}$ was less effective inhibiting the $Ca^{++}$ conductance. If $G \infty c^n$, $c$ stands for concentration of the inhibitor, the slope ($n \simeq -1.0$) of all curves in the straight line region is the same. The inhibition of $Ca^{++}$-conductance by divalent cations (one to one competition) is consistent with the previous assumption that divalent cations compete for the $Ca^{++}$-ionophoric site.

We have reported earlier that $Na^+$ also inhibits $Ca^{++}$-conductance (Shamoo & MacLennan, 1974). Fig. 8 is a representative experiment of conductance versus time in various concentrations of $Na^+$ in the presence

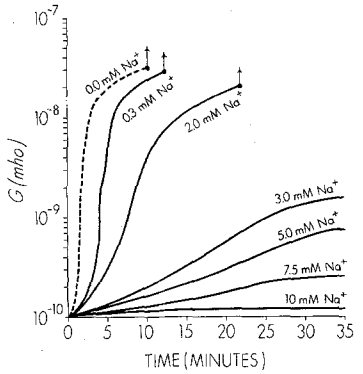


Fig. 8. Effect of sodium chloride on the time response of bilayer conductance in the presence of $5 \times 10^{-4}$ mg/ml $Ca^{++}$-ionophore, 3 mM $CaCl_2$ and 5 mM histidine, pH 7.4

of 3 mM $CaCl_2$ and of the solubilized $Ca^{++}$-ATPase. There are two active regions, one between 0 and 0.2 mM $Na^+$, and the other between 0.2 and 10 mM $Na^+$. The log-log plot of the second region (first region is not a straight line) produces a straight line with $n = -2.2$ or $n = -3.5$ depending on the region taken (i.e. from 0.2 mM to 10 mM or 0.3 mM to 10 mM). This indicates that two sodium ions or above are required to inhibit the $Ca^{++}$-conductance. The fact that $n$ was about $-1.0$ for the divalent inhibitor and about $-2.0$ for the monovalent inhibitor may indicate that the site of competition is the $Ca^{++}$-binding site and that at least two negative charges or their equivalent on the protein must be matched by two positive charges on the metal.

## Discussion

The fall of activity of $(Ca^{++} + Mg^{++})$-ATPase with increased concentrations of methylmercury and mercuric chloride (Figs. 1 and 2) is similar to the inhibitory pattern of mercurial compounds on other enzyme systems such as myosin ATPase and Trypsin (Webb, 1966). The inhibition by the two mercurials indicates that -SH groups are essential for the catalytic function of $(Ca^{++} + Mg^{++})$-ATPase. The coincidence of dose-response curves for the inhibition of $Ca^{++}$ transport and of $(Ca^{++} + Mg^{++})$-ATPase by methylmercury indicates that the hydrolytic function of the enzyme is tightly coupled to the transport function. The data also show that both functions are more sensitive to methylmercuric chloride than to mercuric chloride. One possible mechanism for the differential potency is that $CH_3Hg^+$ has one cationic charge and, therefore, can come closer to the -SH group than can the divalent $Hg^{++}$. This is consistent with the observation that mersalyl inhibits $(Ca^{++} + Mg^{++})$-

ATPase and $Ca^{++}$ transport in a similar fashion to methylmercury, but it is not consistent with the observation that N-ethylmaleimide (an uncharged binder of sulfhydryl group) is no more potent than methylmercury (MacLennan, 1970; Panet & Selinger, 1970).

A more reasonable explanation for the higher sensitivity of both functions to methylmercury than to mercuric chloride is that the target -SH groups are in a slightly hydrophobic environment. $CH_3HgCl$ would have a better chance of reacting with such a sulfhydryl group than $HgCl_2$. This is supported by the observations that NEM inhibits $(Ca^{++}+Mg^{++})$-ATPase and $Ca^{++}$ transport less strongly than methylmercury (Panet & Selinger, 1970), and by the observation that treatment of $(Ca^{++}+Mg^{++})$-ATPase with SDS (sodium deodecylsulfate) increases the number of titratable sulfhydryl groups by four -SH groups per mole of ATPase (Hasselbach & Seraydarian, 1966).

Further evidence for an -SH group in a slightly hydrophobic environment in membrane bound proteins comes from our data on acetylcholine receptor. We found for the acetylcholine receptor from *Torpedo*, that methylmercury at $10^{-6}$ M inhibited 90% of acetylcholine binding to the receptor. In contrast, mercuric chloride at $10^{-3}$ M did not inhibit the acetylcholine binding to the receptor (Shamoo *et al.*, 1975). Moreover, the known sulfhydryl group blockers are not as potent inhibitors of acetylcholine binding to its receptor as methylmercury inhibition (Karlin, 1973).

With mercuric chloride, the active transport of calcium ions is more susceptible to inhibition than is the $(Ca^{++}+Mg^{++})$-ATPase activity ($^{K}0.5$ $V_{max}=8\times10^{-5}$ M $HgCl_2$ for transport, where $^{K}0.5$ $V_{max}=1.1\times10^{-4}$ M $HgCl_2$ for ATPase). This is explained in terms of a summation of two independent actions of $Hg^{++}$ on the transport system: the inhibition at the enzyme site and the inhibition at the ionophoric site. The evidence that methylmercury does not act on the ionophore is thus both direct (from black membrane experiment) and indirect (from the coincidence of dose-response curves discussed in the preceding paragraph). The evidence indicates that the $Ca^{++}$ ionophore has no sulfhydryl groups essential for its activity. Additional evidence for this point of view is that the 20,000 mol wt fragment of the ATPase which is rich in ionophoric activity (Shamoo & Ryan, 1975) does not contain any cysteine residues (Stewart & MacLennan, 1975). Thus, our data indicate that the $(Ca^{++}+Mg^{++})$-ATPase enzyme has one site for the $Ca^{++}$ ionophore and a different site for ATP hydrolysis. The proximity and relationship of the two sites will shed light on the energy transduction mechanism.

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