Activation of Ca<sup>2+</sup> Uptake and Inhibition of Reversal of the Sarcoplasmic Reticulum Ca<sup>2+</sup> Pump by Aromatic Compounds

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The effects of aromatic compounds in sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase were investigated. The solubility of the drugs in various organic solvents and water was measured. The ratio between the solubility in organic solvents and that in water (distribution coefficient) was used as an index of their hydrophobicity. The order found was triphenylphosphine > phenylamine > 3-nitrophenol > 4-nitrophenol > 1,3-dihydroxybenzene. The effects observed on the Ca<sup>2+</sup>-ATPase were correlated with hydrophobicity of the drugs, activation and inhibition being obtained at a lower concentration the greater the distribution coefficient of the drug into organic solvent.

In leaky vesicles, the effects of each compound on the ATPase activity varied depending on the Ca<sup>2+</sup> concentration in the medium: it inhibited in the presence of 5 μM Ca<sup>2+</sup> and activated when the Ca<sup>2+</sup> concentration was raised to 2 mM. In intact vesicles, 3- and 4-nitrophenol, diphenylamine, and triphenylphosphine enhanced both the rate of ATP hydrolysis and the amount of Ca<sup>2+</sup> accumulated by the vesicles. These four drugs inhibited Ca<sup>2+</sup> uptake when ITP was used as substrate. 1,3-Dihydroxybenzene enhanced the amount of Ca<sup>2+</sup> accumulated by the vesicles regardless of whether ATP or ITP was the substrate.

All five compounds inhibited the phosphorylation of the enzyme by Pi, the efflux of Ca<sup>2+</sup>, and the synthesis of ATP measured during the reversal of the Ca<sup>2+</sup>-pump. The results indicate that the hydrophobic character of various organic compounds determines their access to sensitive domains of the membrane-bound calcium pump. Additional specific effects are then produced, depending on the structure of each compound.

The Ca<sup>2+</sup>-ATPase of sarcoplasmic reticulum can catalyze both the hydrolysis and the synthesis of ATP. During the hydrolysis of ATP, Ca<sup>2+</sup> is accumulated by the vesicles. In the reverse process, the energy derived from the Ca<sup>2+</sup>-gradient is used by the ATPase to synthesize ATP from ADP and Pi, the efflux of Ca<sup>2+</sup>, and the synthesis of ATP being coupled to a fast release of Ca<sup>2+</sup>.

The Ca<sup>2+</sup>-ATPase activities of the sarcoplasmic reticulum ATPase. The alterations reported so far, however, do not appear to fit a common pattern, as both activation and inactivation of various partial reactions of the catalytic and transport cycle have been observed. We have now chosen five compounds that encompass a wide range of hydrophobicity, measured their distribution coefficients, and studied their effects on transport and ATPase functions.

The data presented are discussed according to the reaction sequence shown in Fig. 1 (Refs. 1, 3, and 5).

MATERIALS AND METHODS

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle as described by Eletre and Inesi (20). The Ca<sup>2+</sup>-ATPase was purified from the vesicles according to MacLennan (21) and Meissner et al. (22). Both preparations have a high ATPase activity but are unable to accumulate Ca<sup>2+</sup>.

<sup>32</sup>P, was obtained from the Brazilian Institute of Atomic Energy and purified as described previously (23). ATPase activity was assayed by measuring the release of <sup>32</sup>P from (1<sup>13</sup>PH<sub>2</sub>) ATP (24).

Calcium uptake and efflux were measured by the filtration method using Millipore filters (25).

Phosphorylation of the Ca<sup>2+</sup>-ATPase by <sup>32</sup>P, was assayed as described previously and corrected for nonspecific binding (26). Synthesis of ATP was assayed by measuring the formation of [γ<sup>32</sup>P]<sub>ATP</sub> from ADP and <sup>32</sup>P, (27).

The drug solutions used were freshly prepared in dimethyl sulfoxide or ethanol. The final concentration of dimethyl sulfoxide in the medium is given in the text, and the final concentration of ethanol was equal or inferior to 2% (v/v).

The solubility of each aromatic compound was determined using a saturated solution of the drug. This solution was prepared by incubating an excess of the drug with 2 ml of the solvent at 35 °C for several h, with occasional shaking. Then, the temperature of the solution was decreased to 25 °C. This promoted the formation of crystals that were centrifuged at 2500 rpm for 20 min at 25 °C. An aliquot of the supernatant was diluted serially from 10 to 10<sup>6</sup>-fold in the same solvent, and the concentration of dissolved material was calculated by comparing these optical densities with standard curves, as follows. The absorption spectra of freshly prepared standard solutions were determined in the different solvents used (H<sub>2</sub>O, 10% dimethyl sulfoxide, 100% Me<sub>2</sub>SO, isobutyl alcohol, methanol, benzene), and extinction coefficients at the wavelength of maximal absorbance were calculated. The distribution coefficients (Table I) were calculated by dividing the solubility of each compound in 100% dimethyl sulfoxide, isobutyl alcohol, methanol, and benzene by the respective solubility in either H<sub>2</sub>O or 10% dimethyl sulfoxide (27).

We were not able to measure the partitioning of the aromatic compounds into the membranes of the sarcoplasmic reticulum vesicles. However, the concentration dependence of enzyme inhibition (K<sub>i</sub>) by the various compounds was obtained from the values of apparent K<sub>app</sub> for P<sub>i</sub> measured in the absence and in the presence (K<sub>app</sub>) of each inhibitor (I), according to the equation K<sub>app</sub> = K<sub>app</sub><sup>-1</sup> + 1/K<sub>i</sub>.

RESULTS

Ca<sup>2+</sup> Uptake and ATPase Activity—When ATP was used as substrate, all the compounds tested promoted an increase...
Fig. 1. The catalytic cycle of Ca\textsuperscript{2+}-ATPase. The sequence includes two distinct functional states of the enzyme, E and \textsuperscript{*}E. The Ca\textsuperscript{2+}-binding sites in the E form face the external surface of the vesicles and have a high affinity for Ca\textsuperscript{2+} (\(k_a = 10^{-8} \text{ M}\) at pH 7.0). In the \textsuperscript{*}E form, the Ca\textsuperscript{2+}-binding sites face the lumen of the vesicles and have a low affinity for Ca\textsuperscript{2+} (\(k_a = 10^{-3} \text{ M}\)).

TABLE I
Hydropobicity of the compounds tested

<table>
<thead>
<tr>
<th>Compound</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHB</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>pNP</td>
<td>45</td>
<td>50</td>
<td>48</td>
<td>111</td>
<td>1</td>
</tr>
<tr>
<td>mNP</td>
<td>45</td>
<td>50</td>
<td>57</td>
<td>81</td>
<td>2</td>
</tr>
<tr>
<td>DPA</td>
<td>6,636</td>
<td>12,167</td>
<td>5,233</td>
<td>7,832</td>
<td>44,875</td>
</tr>
<tr>
<td>TPP</td>
<td>16,471</td>
<td>186,667</td>
<td>6,842</td>
<td>9,256</td>
<td>164,260</td>
</tr>
</tbody>
</table>

![Fig. 2](image)

Calcium uptake. The assay medium composition was 50 mM MOPS/Tris buffer (pH 7.0), 0.06 mM \(\text{CaCl}_2\), 80 mM KCl, 1 mM P, 10 mM MgCl\(_2\), and either 1 mM ATP (●) or 1 mM ITP (○). The reaction was started by the addition of vesicles to a final concentration of 0.02 mg of protein/ml and was arrested by Millipore filtration after a 30-min incubation at 35 °C.

in Ca\textsuperscript{2+} uptake (Fig. 2, filled circles). The effect was biphasic, the activation being abolished when an excess of the compound was used. The concentration needed for maximal activation of Ca\textsuperscript{2+} uptake was lower the more hydrophobic the compound tested (compare Table I and Fig. 2). However, the extent of enhancement of Ca\textsuperscript{2+} uptake at the optimal concentration of each compound followed a different pattern, as the enhancement by mNP, pNP, and TPP was more pronounced than that of DPA and DHB. The concentration profile for pNP (not shown) was the same as that for mNP.

When ITP was used as substrate (instead of ATP), DHB promoted a small but significant activation of uptake. The other compounds tested did not activate Ca\textsuperscript{2+} uptake, even at concentrations of drug 50-fold smaller than those shown in Fig. 2 (data not shown). On the contrary, all of them inhibited uptake at higher concentrations (Fig. 2, empty circles).

To examine the effects of the drugs on ATPase activity, two enzyme preparations were used: intact vesicles, and a purified Ca\textsuperscript{2+}-ATPase (Fig. 3). In intact vesicles, the Ca\textsuperscript{2+} accumulated during ATP hydrolysis caused the Ca\textsuperscript{2+} concentration inside the vesicles to increase to levels that were much higher than that found outside the vesicles. Both preparations of purified ATPase used have been shown to form leaky vesicles, so that the Ca\textsuperscript{2+} concentration was the same on both sides of the membrane (21, 22). The five compounds tested inhibited the ATPase activity when the purified ATPase was used, and the Ca\textsuperscript{2+} concentration in the medium was sufficient to bind only to the high affinity Ca\textsuperscript{2+}-binding site (Fig. 3, empty circles). The concentration profile for pNP (not shown) was the same as that for mNP. The concentration of drug needed for half-maximal inhibition of ATPase activity was smaller the higher its partition coefficient (compare Table I and Fig. 3).

A different pattern was observed when both the high- and low affinity Ca\textsuperscript{2+}-binding sites of the enzyme were saturated. Under this condition, the ATPase activity was enhanced by mNP, DPA, and TPP. This was observed with the use of either intact vesicles in the presence of 5 \(\mu\text{M}\) Ca\textsuperscript{2+} (Fig. 3, × symbols) or purified Ca\textsuperscript{2+}-ATPase incubated in the presence of 2 \(\mu\text{M}\) Ca\textsuperscript{2+} (Fig. 3, filled circles). The effect was biphasic, the hydrolysis of ATP reaching a maximum and then decreasing as the concentration of each compound was raised. The concentrations needed for maximal increase of ATP hydrolysis (Fig. 3) were in the same range as those needed for maximal increase of Ca\textsuperscript{2+} uptake (Fig. 2). Different results were obtained with DHB (Fig. 3A). In intact vesicles, this compound either had no effect or promoted a small increase (5–10%) in the rate of ATP hydrolysis.

Reversal of the Ca\textsuperscript{2+} Pump—Both active Ca\textsuperscript{2+} efflux and

1The abbreviations used are: mNP, 3-nitrophenol; EGTA, (ethylenethylenenitrilo)tetraacetic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; DHB, 1,3-dihydroxybenzene; pNP, 4-nitrophenol; DPA, diphenylamine; TPP, triphenylphosphine.
synthesis of ATP were inhibited by the different compounds tested (Figs. 4 and 6A, and Table II). The passive Ca²⁺ efflux was not increased (Table II), indicating that the inhibition of ATP synthesis was not caused by ionophoric effects, which would dissipate the Ca²⁺ gradient. The drugs were effective as inhibitors in the same order as their distribution coefficients (compare Fig. 4 and Table I).

The first step in the reversal of the Ca²⁺ pump is the enzyme phosphorylation of P, (steps 6 and 7 in Fig. 1). The five compounds inhibited this reaction. This was observed with a seemingly competitive pattern with respect to P, (Fig. 6) in totally aqueous medium (data not shown) and in the presence of 10% dimethyl sulfoxide (Fig. 6) when either leaky vesicles (Fig. 6) or intact vesicles previously loaded with calcium were used (Fig. 5A). The Kᵢ values for DHB, pNP, mNP, DPA, and TPP measured in totally aqueous medium were 3.2, 3.5,

![Inhibition of ATP synthesis](Fig. 4)

The vesicles were loaded with Ca²⁺ in a medium containing 60 mM MOPS/Tris buffer (pH 6.5), 100 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 1 mM CaCl₂, 1 mM ITP, 1 mM CaCl₂, 1 mM EGTA, 10% dimethyl sulfoxide, 2 mM oxalate, and 0.5 mg/ml of vesicle protein. The mixture was centrifuged at 60,000 g for 40 min. The pellet was stored on ice and resuspended in water before use. After resuspension, the amount of calcium inside the vesicles varied in the different experiments between 0.8 and 0.9 nmol of calcium/mg of protein.

The assay medium was 50 mM MOPS/Tris buffer (pH 6.5), 20 mM MgCl₂, 0.3 mM CaCl₂, 80 mM KCl, 10 mM acetyl phosphate, and sarcoplasmatic reticulum vesicles to a final concentration of 0.3 mg/ml. After a 30-min incubation at 35°C, the mixture was centrifuged at 60,000 × g for 40 min. The pellet was stored on ice and resuspended in water before use. After resuspension, the enzyme phosphorylation of the enzyme by Pi (Figs. 4 and 6) in the presence of 10% dimethyl sulfoxide (Fig. 6), they were 9.5, 1.4, 0.6, 0.2, and 0.04 mM, respectively.

A surprising finding was that in contrast to phosphorylation by Pᵢ (Figs. 5A and 6), the inhibition of ATP synthesis could not be reversed by raising the Pᵢ concentration of the medium. This is shown in Fig. 6B for mNP; a similar inhibition of ATP synthesis was observed when pNP, DPA, and TPP were used (data not shown). This finding indicates that the compounds used were able to modify partial reactions other than those involved in the phosphorylation of the enzyme by Pᵢ.
**Ca^{2+}-ATPase, Aromatic Compounds**

**DISCUSSION**

The good correlation between the partition coefficients of the aromatic compounds tested (Table I) and the concentration dependence of various effects tested (Figs. 2-6) suggest that the compounds interact with hydrophobic domains of the calcium pump. At present, we do not know whether these are hydrophobic portions of the protein or simply the lipid moiety of the membrane. On the other hand, the extent of enhancement of Ca^{2+} uptake varied depending on the compound used, mNP and TPP being more effective than DBH and DPA. This indicates that the hydrophobicity of the compounds plays an important role in determining their access to a susceptible region of the pump; then, steric factors and the chemical structure of the compounds determine the intensity of the effect.

Previous studies have shown that the increase in Ca^{2+} concentration within the vesicles which occurs following brief incubation impairs the accumulation of Ca^{2+} in subsequent incubation intervals, promotes a decrease in the steady-state rate of ATP hydrolysis (28, 29), and activates the synthesis of ATP from ADP and P, (30–32). These effects are associated with the binding of Ca^{2+} to a site on the enzyme which faces the vesicles’ lumen (1–5, 33). The findings that the compounds tested increase the amount of Ca^{2+} accumulated by the vesicles (Fig. 2), enhance the ATPase activity of intact vesicles (Fig. 3), and inhibit the synthesis of ATP measured during reversal of the Ca^{2+} pump (Fig. 4) suggest that they impair the effect derived from the binding of Ca^{2+} to the low affinity binding site of the enzyme.

The ATPase possesses two classes of binding sites for ATP: a high affinity catalytic site, and a second, low affinity regulatory site at which binding of ATP accelerates the turnover of the enzyme (1–6). ITP does not share this latter property; it binds only to the catalytic site and not to the regulatory site (1, 3). The finding that the ITP-driven Ca^{2+} uptake was inhibited by the same concentrations of aromatic compounds which activate Ca^{2+} uptake supported by ATP (Fig. 2) raises the possibility that the effects on Ca^{2+} uptake and on the low affinity Ca^{2+}-binding site are related to the mechanism by which ATP regulates the enzyme. Evidence obtained in different laboratories suggests that the environment of the catalytic site of the Ca^{2+}-ATPase may undergo a hydrophobic-hydrophilic transition (34–41). In the E form, the catalytic site would have a hydrophilic character; in the *E* form, it would be hydrophobic. The good correlation found between the *K* values and the distribution coefficients of all the compounds tested (Table 1 and Fig. 6) suggests that the hydrophobic compounds partition into the catalytic site of the *E* form, impairing the entry of P, (steps 6 and 7 in Fig. 1). The inhibition of ATP synthesis, however, cannot be explained only by a competition of the drugs with P. The finding that the inhibition of ATP synthesis is not overcome by high P, concentrations (Fig. 5B) indicates that in addition to competing with P, the compounds also impair phosphoryl transfer from the phosphoenzyme in ADP.

**Correlation with Other Studies**—Various investigators have tested the effects of hydrophobic molecules on the Ca^{2+}-ATPase of sarcoplasmic reticulum. The effects observed seem to vary depending on the experimental conditions used. In early reports, it was thought that diethyl ether was only able to permeabilize the membrane of the vesicles and thus to inhibit Ca^{2+} uptake (6, 42, 43). Later, it was shown that diethyl ether can increase both the ATPase activity and the amount of Ca^{2+} accumulated by the vesicles; the effect of the solvent depended on its concentration and on whether or not the vesicles were centrifuged (7–9). Recently (12), it was shown that dimethyl sulfoxide has an effect similar to that shown in Fig. 2; it increases Ca^{2+} uptake when ATP is used, and it inhibits when ITP is used as substrate.

Melgunov et al. (10) reported that low concentrations of alkylate promote a small activation of Ca^{2+} uptake, varying from 10 to 50%, and that higher concentrations inhibit the activity of the Ca^{2+} pump. The activity of each alkylate tested varied with its partition coefficient. These authors did not explore the effects of these drugs on reversal of the Ca^{2+} pump. Wakabayashi et al. (19) observed that 3,3′,4′,5-tetrasalicylanilide enhances calcium uptake and ATPase activity of sarcoplasmic reticulum vesicles. This effect was observed only at 6°C and was abolished when the temperature was raised to the range of 25–35°C. At high concentrations, the drug inhibited the enzyme regardless of the temperature of the medium. Jones et al. (14) studied the effects of hexachlorocyclohexanes on the ATPase reconstituted into bilayers. These compounds either activated or inhibited the enzyme depending on the phospholipid used to form the bilayers. An effect similar to that shown in Fig. 3 was observed by Sokolove et al. (17) using different phenolic antioxidants. These compounds inhibited the ATPase activity of leaky vesicles in the presence of low Ca^{2+} concentrations but enhanced the ATPase activity when Ca^{2+} interacted with the low affinity form of the Ca^{2+}-binding site. Suko et al. (15) reported that Tetracaine and Nupercaine, two local anesthetics, inhibited both the Ca^{2+} uptake and reversal of the Ca^{2+} pump. These authors did not explore a possible competition with P, and attributed the effect observed to a partitioning of the drugs into the lipid moiety of the membrane. Finally, a 165-kDa protein was recently purified from sarcoplasmic reticulum vesicles (44). This protein is located in the vesicles’ lumen and is able to bind both Ca^{2+} and plasma lipoproteins. The possibility remains that the different hydrophobic drugs tested may interact on the same binding region of the membrane on which this protein interacts.

All of these reports, as well as our present studies, indicate that the hydrophobic character of a large number of compounds determines their access to the Ca^{2+}-dependent ATPase, which is the operator of the pump. However, various partial reactions of the catalytic and transport cycle are affected in a specific way by each compound.

**REFERENCES**