Local Anesthetics Induce Fast Ca\(^{2+}\) Efflux through a Nonenergized State of the Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase*

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The effect of the local anesthetics SKF 525-A, dibucaine, tetracaine, procaine, and benzocaine on sarcoplasmic reticulum vesicles was studied. All the anesthetics tested inhibited the phosphorylation of the Ca\(^{2+}\)-ATPase by Pi, in a competitive manner. Tertiary amine and positively charged anesthetics, in addition to competing with Pi, also decreased the apparent affinity of the ATPase for Mg\(^{2+}\). There was a good correlation between the octanol/water partition coefficients and the inhibitory activity of the different anesthetics.

All the anesthetics tested induced a 5- to 10-fold increase in the rate of Ca\(^{2+}\) efflux. This was promoted by the same drug concentration that inhibited the phosphorylation of the ATPase by Pi. The effect on Ca\(^{2+}\) efflux was antagonized by the ligands of the ATPase (Mg\(^{2+}\), K\(^{+}\), Ca\(^{2+}\), MgATP, and ADP) and by the organic polyamines ruthenium red, spermine, spermidine, and putrescine. The natural anion heparin was found to potentiate the effect of the positively charged anesthetics on the rate of Ca\(^{2+}\) efflux.

It is concluded that the local anesthetics increase the Ca\(^{2+}\) efflux through a nonenergized state of the Ca\(^{2+}\)-ATPase, rather than promoting a nonspecific Ca\(^{2+}\) leakage through the membrane.

The Ca\(^{2+}\)-ATPase of sarcoplasmic reticulum is a membrane-bound protein that is responsible for the active Ca\(^{2+}\) accumulation that occurs during muscle relaxation (1, 2). During Ca\(^{2+}\) uptake, the enzyme is phosphorylated by ATP and generates a Ca\(^{2+}\) gradient across the membrane. In the reverse process, the ATPase is phosphorylated by Pi, and the energy derived from the Ca\(^{2+}\) gradient is used by the ATPase to synthesize ATP from ADP and Pi. The synthesis of ATP is coupled to a fast efflux of Ca\(^{2+}\) (1).

Two classes of vesicles can be separated by sucrose density gradient centrifugation (3–5). These are the "heavy" or "junctional" vesicles, which are enriched in terminal cisternae, and the "light" vesicles, which are mainly derived from longitudinal tubules. The light vesicles do not contain the ryanodine/caffeine-sensitive Ca\(^{2+}\) channels found in the junctional vesicles (6, 7).

A Ca\(^{2+}\) efflux that is mediated by the ATPase and is not coupled with the synthesis of ATP is observed when light vesicles are incubated in media containing none of the substrates of the ATPase (8). The rate of this efflux is similar to that measured during reversal of the Ca\(^{2+}\) pump and the efflux is arrested when the enzyme is phosphorylated by Pi. Different drugs that inhibit phosphorylation of the ATPase by Pi are also able to activate the Ca\(^{2+}\) efflux mediated by the ATPase (9). This includes propranolol, a tertiary amine that at high concentrations has local anesthetic properties.

Local anesthetics such as tetracaine, dibucaine, SKF 525A, procaine, and benzocaine are known to modify the rate of Ca\(^{2+}\) efflux measured in sarcoplasmic reticulum vesicles (10–20). The effect of these anesthetics varies depending on the drug concentration and experimental condition used. At low concentrations they decrease the rate of Ca\(^{2+}\) efflux (10–16) while at high concentrations they increase it (17–20). The increment of Ca\(^{2+}\) efflux has been attributed to nonspecific leakage through the membrane (17–20). On the basis of the data obtained in a previous report with hydrophobic drugs (9), we raise the possibility that the effect of different local anesthetics on the rate of Ca\(^{2+}\) efflux may not be related to nonspecific interaction with the lipid moiety of the membrane but rather, to a direct effect on the Ca\(^{2+}\) transport ATPase. In this report this possibility was confirmed, using the same rationale and experimental approach previously used to study the effect of propranolol and of other hydrophobic molecules on the Ca\(^{2+}\)-ATPase of sarcoplasmic reticulum (9).

MATERIALS AND METHODS

Light sarcoplasmic reticulum vesicles were isolated from rabbit skeletal muscle as described by Eletr and Inesi (21) and stored in liquid nitrogen. As previously shown (22), electrophoretic analysis of the preparations revealed a low content of calsequestrin and practically no junctional proteins. Protein concentration was determined according to Lowry et al. (23).

\(^{32}\)P was obtained from the Brazilian Institute of Atomic Energy and purified as previously described (24).

Phosphorylation of the Ca\(^{2+}\)-ATPase by \(^{32}\)P, was assayed as described previously and corrected for nonspecific binding (25).

For the Ca\(^{2+}\) efflux experiments, the vesicles were preloaded with \(^{45}\)Ca in a medium containing 50 mM MOPS-Tris, pH 7.0, 10 mM MgCl\(_2\), 20 mM Pi, 0.3 mM CaCl\(_2\), 2 mM ATP, and 0.05 mg of vesicle protein/ml. After 30 min, at 25 °C, the vesicles were sedimented by centrifugation at 40,000 \(\times\) g for 20 min, the supernatant was discarded, and the walls of the tubes were blotted to minimize the volume of residual loading medium. The pellets (0.8 mg each) were kept in ice and resuspended in 1.6 ml of cold water with three strokes of a glass homogenizer. The suspension was diluted into efflux media to a final concentration of 0.05 mg of vesicle protein/ml. The loaded vesicles were never left in water for more than 2 min before dilution into the efflux medium. The contaminant Pi derived from the loading mixture never exceeded a final concentration of 40 µM in the efflux medium (9).

The free Ca\(^{2+}\) concentration was calculated using the apparent

1 The abbreviations used are: MOPS, 4-morpholinopropanesulfonic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid.

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association constants for Ca/EGTA provided by Orentlicher et al. (26) using a computer program as described by Fabiato and Fabiato (27).

Benzocaine solution was freshly prepared in ethanol. The final ethanol concentration in the assay media did not exceed 1% (v/v). All other anesthetics were freshly dissolved in water. Ruthenium red, heparin (grade I, with molecular weight between 6,000 and 12,000), and all other reagents were obtained from Sigma and used without further purification. Ca was purchased from Du Pont-New England Nuclear (Wilmington, DE).

The experiments shown in the figures were repeated at least three times.

RESULTS

Phosphorylation of the Enzyme by Pi—In previous reports (9, 28–30), it was shown that both the phosphorylation of the ATPase by Pi, and the synthesis of ATP measured during the reversal of the Ca pump are inhibited by different hydrophobic molecules. Inhibition is overcome by excess Pi, indicating that hydrophobic molecules compete with Pi for entry into the catalytic site.

We now show that the local anesthetics SKF 525-A, dibucaine, tetracaine, benzocaine, and procaine inhibit phosphorylation of the ATPase by Pi in a competitive manner (Fig. 1). With the exception of benzocaine, all the anesthetics tested are positively charged at pH 6.0 (Table I). The formulas of these anesthetics are shown in Ref. 16. In this reference the formula of tetracaine was misprinted, the correct formula being:

\[
\text{C}_9\text{H}_{15}\text{N} \rightarrow \text{C} - \text{O} - \text{C}_2\text{H}_3 - \text{N} \rightarrow \text{C}_3\text{H}_7
\]

Phosphorylation by Pi occurs only in the presence of Mg, but the true substrate for phosphoprotein formation is free Pi, and not the complex Mg.Pi (31–35). In the presence of 1 mM Pi, the positively charged anesthetics were able to compete with Mg (Figs. 2, A and B, and 3A). The neutral anesthetic benzocaine exhibited a noncompetitive inhibition with respect to Mg (Figs. 2C and 3B).

Fig. 4 shows that there is a good correlation \((r = -0.881)\) between the octanol/water partition coefficients of the local anesthetics and their ability to inhibit the phosphorylation reaction. Similar results have been reported for other hydrophobic drugs (30). Note in Table I that the positively charged anesthetics have practically the same ability to compete with Pi as they do with Mg.

<table>
<thead>
<tr>
<th>Drug</th>
<th>pK(_A)</th>
<th>Free base</th>
<th>F</th>
<th>K(_P)</th>
<th>K(_{Mg})</th>
<th>A(_{100})</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKF 525-A</td>
<td>8.8</td>
<td>0.16</td>
<td>44,668 (60)</td>
<td>0.06</td>
<td>0.08</td>
<td>0.18</td>
</tr>
<tr>
<td>Dibucaine</td>
<td>8.5</td>
<td>0.31</td>
<td>5,000 (61)</td>
<td>0.27</td>
<td>0.29</td>
<td>0.25</td>
</tr>
<tr>
<td>Tetracaine</td>
<td>8.5</td>
<td>0.31</td>
<td>2,512 (61)</td>
<td>3.04</td>
<td>2.50</td>
<td>1.10</td>
</tr>
<tr>
<td>Procaine</td>
<td>8.9</td>
<td>0.12</td>
<td>100 (61)</td>
<td>50.00</td>
<td>28.00</td>
<td>40.00</td>
</tr>
<tr>
<td>Benzocaine</td>
<td>2.6</td>
<td>100.00</td>
<td>41 (60)</td>
<td>3.11</td>
<td>6.00</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Mg\(^{2+}\) dependence of inhibition of phosphoenzyme formation by local anesthetics. The conditions were the same as described in the legend to Fig. 1, except that the Pi concentration was fixed at 1 mM and the MgCl\(_2\) concentration was varied between 2 and 20 mM. A: control (○), 2 mM tetracaine (●), 0.3 mM dibucaine (△); B: control (○), 80 μM SKF 525-A (■), 100 mM procaine (▲); C: control (○), 2 (●) and 4 (△) mM benzocaine.

Fig. 3. Concentration dependence of tetracaine (A) and benzocaine (B) inhibition of phosphoenzyme formation, and protection by Mg\(^{2+}\). The conditions were the same as described in the legend to Fig. 1. The reaction media contained 50 mM MOPS-Tris (pH 6.0), 5 mM EGTA, 1 mM Pi, and either 2 (○) or 20 (●) mM MgCl\(_2\).

anesthetics have practically the same ability to compete with Pi as they do with Mg\(^{2+}\).

Passive Ca\(^{2+}\) Efflux—When vesicles preloaded with Ca\(^{2+}\) are diluted in a medium containing EGTA (Ca\(^{2+}\)-free medium), the internal Ca\(^{2+}\) is slowly released (0.2–0.5 μmol/mg-min). In the presence of low Pi, and Mg\(^{2+}\) concentrations, all the anesthetics tested increased the rate of Ca\(^{2+}\) efflux, reaching rates higher than 2 μmol/mg-min (Figs. 5–7). In the absence of ligands of the ATPase (non-energized state), the concentration of anesthetic required to double the rate of Ca\(^{2+}\) efflux was in the same range as that required to compete with Pi or Mg (Table I). Like the K\(_I\) for the...
phosphorylation reaction, the $A_{100}$ is strongly correlated with hydrophobicity of each drug tested ($r = -0.891$, Table I).

Addition of Mg$^{2+}$ (10 mM), Ca$^{2+}$ (50 mM), or K$^{+}$ (100 mM) greatly decreased the effects of the drugs on Ca$^{2+}$ efflux (Figs. 5-7). These ions promoted shifts in the anesthetic concentrations required to enhance Ca$^{2+}$ efflux (Figs. 6 and 7), and in some cases also attenuated the maximum effect of the drugs (Fig. 8). The relative potencies of the cations for antagonizing the effects of the drugs on Ca$^{2+}$ efflux followed the order: Ca$^{2+} >$ Mg$^{2+} >$ K$^+ >$ Na$^+$ > Li$^+$. Lithium was only half as effective as K$^+$, and many times less effective than Ca$^{2+}$ (Fig. 8), indicating that ionic strength is not a determinant factor for the effect of the cations. The addition of Na$^+$ produced the same effect as K$^+$ (data not shown). Although all the anesthetics tested competitively inhibited the phosphorylation by P, (Fig. 1), the addition of 4 mM P, to the efflux medium did not significantly modify the Mg$^{2+}$ concentration needed to decrease the rate of Ca$^{2+}$ efflux (data not shown). The cation concentrations that antagonized the effects of the local anesthetics were in the same concentration range as that needed for binding to the Ca$^{2+}$-ATPase.

**Fig. 4.** Correlation between hydrophobicity and inhibition of phosphoenzyme formation by local anesthetics. The values were obtained from the octanol/water partition coefficients (F) and $K_i$ values shown in Table I for competition with both P, (O) and Mg$^{2+}$ (C).

**Fig. 5.** Enhancement of Ca$^{2+}$ efflux by tetracaine and SKF 525-A; effects of external Mg$^{2+}$ and Ca$^{2+}$. Light sarcoplasmic reticulum vesicles were actively loaded with Ca$^{2+}$ as described under "Materials and Methods." The maximum of Ca$^{2+}$ loading was measured by diluting the vesicles in an efflux-blocking medium, containing 50 mM MOPS-Tris (pH 7.0) and 300 mM La(NO$_3$)$_3$. For the Ca$^{2+}$ efflux experiments, the vesicles were diluted to 0.05 mg of protein/ml final concentration into an efflux medium containing 50 mM MOPS-Tris (pH 7.0), 5 mM EGTA, 0.1 mM P$_i$, and 0.1 mM MgCl$_2$ (A), 10 mM MgCl$_2$ (B), or 0.5 mM free Ca$^{2+}$ concentration, by a Ca/EGTA buffer (C). Control ( ), 2 mM tetracaine ( ), or 0.2 mM SKF 525-A (o). After dilution, at the times indicated in the abscissa, the vesicles were quickly filtered through Millipore filters (0.45 μm) and washed 3 times with 3 mM La(NO$_3$)$_3$.

**Fig. 6.** Concentration dependence for tetracaine (A), dibucaine (B), and SKF 525-A (C) in enhancing Ca$^{2+}$ efflux with different cations present. The conditions were the same as described in the legend to Fig. 5. The efflux medium contained 50 mM MOPS-Tris (pH 7.0), 5 mM EGTA, 0.1 mM P$_i$, and 0.1 mM MgCl$_2$ (O), 10 mM MgCl$_2$ ( ), 0.1 mM MgCl$_2$, and 100 mM KCl (Δ) or 0.1 mM MgCl$_2$ and 50 μM free Ca$^{2+}$ (∆). The efflux time was 1 min and the maximum of Ca$^{2+}$ loading varied between 2 and 2.2 μmol of Ca$^{2+}$/mg of protein.

**Fig. 7.** Concentration dependence for procaine (A) and benzocaine (B) enhancement of Ca$^{2+}$ efflux. The conditions and symbols are identical to Fig. 6, except that the efflux time was 2 min.

**Fig. 8.** Concentration dependence for Ca$^{2+}$, Mg$^{2+}$, K$^+$, and Li$^+$ blocking effects. The efflux medium contained 50 mM MOPS-Tris (pH 7.0), 5 mM EGTA, 0.1 mM P$_i$, 2 mM tetracaine, and various cation concentrations. A, Ca$^{2+}$ efflux as a function of free Ca$^{2+}$ (O) or MgCl$_2$ concentration ( ). B, Ca$^{2+}$ efflux as a function of KCl ( ) and LiCl ( ) concentration.

This supports the proposal that the Ca$^{2+}$ efflux promoted by anesthetics is mediated by the Ca$^{2+}$ pump and is not due to a nonspecific leakage or disruption of the membrane, as proposed by others (17-20).

**Effects of Organic Polyamines**—Ruthenium red and spermidine both reduce the Ca$^{2+}$ efflux from the light fraction of sarcoplasmic reticulum and compete with Ca$^{2+}$, Mg$^{2+}$, and K$^+$ for their effect on the enzyme (9, 28, 36). Spermine, spermidine, and putrescine are polyamines found in different animal tissues, including skeletal muscle (37). We now show that the organic polyamines block the effect of tetracaine (Figs. 9 and 10). In the presence of 2 mM tetracaine, their relative poten-
that for ATP and ADP (1). Addition of 1 mM of either ITP or IDP to the efflux medium produced only a slight inhibition of Ca²⁺ efflux (data not shown). AMP and acetylphosphate up to a concentration of 1 mM, had no effect on the increment of Ca²⁺ efflux promoted by tetracaine (data not shown).

**Fig. 9. Effects of ruthenium red and spermidine on tetracaine-induced Ca²⁺ efflux.** The efflux medium contained 50 mM MOPS-Tris (pH 7.0), 5 mM EGTA, 0.1 mM P₃, 0.1 mM MgCl₂, and no addition (C), 70 μM ruthenium red (○), or 4 mM spermidine (△). The maximum of Ca²⁺ loading was 1.6 μmol/mg of protein. Other conditions were the same as the control in Fig. 6.

**Fig. 10. Concentration-dependent block of Ca²⁺ efflux by organic polyamines.** The efflux medium contained 2 mM tetracaine, 50 mM MOPS-Tris (pH 7.0), 5 mM EGTA, 0.1 mM P₃, 0.1 mM MgCl₂, and various concentrations of spermidine (△), spermine (○), or putrescine (Δ). The maximum of Ca²⁺ loading was 3.2 μmol/mg of protein.

**Fig. 11. Inhibition of Ca²⁺ efflux by ATP and ADP; effect of Mg²⁺.** A, the efflux medium contained 50 mM MOPS-Tris (pH 7.0), 5 mM EGTA, 0.1 mM P₃, and no addition (C), 0.5 mM ATP (○), or 0.5 mM ADP (△). B, Ca²⁺ efflux in the presence of 2 mM tetracaine as a function of ATP concentration. C, Ca²⁺ efflux in the presence of 2 mM tetracaine as a function of ADP concentration. In B + C the efflux was carried out in the absence of Mg²⁺ (C, 0.5 mM EDTA) or in the presence of 0.2 mM free Mg²⁺ (●, 0.5 mM EDTA).

**Fig. 12. Potentiation of tetracaine effect by heparin.** The efflux medium contained 50 mM MOPS-Tris (pH 7.0), 5 mM EGTA, 0.1 mM P₃, 0.1 mM MgCl₂, and (A) either 0 (○) or 10 μg/ml of heparin (●), as well as the tetracaine concentration on the abscissa. B, Ca²⁺ efflux as a function of heparin concentration in the presence of 0 (C), 0.5 (△), or 1 (○) mM tetracaine. The maximum of Ca²⁺ loading varied between 1.6 (●) and 2 (○) μmol/mg of protein.

**DISCUSSION**

Suko et al. (18) showed that tetracaine and dibucaine inhibit both the reversal of the Ca²⁺ pump and the phosphorylation of the ATPase by either P₃ or ATP. These effects were attributed to binding of local anesthetics to the phospholipids of the membrane (17-19) and to changes in the protein-lipid interface (41). The competitive kinetics shown in Figs. 1-3 indicate that the local anesthetics may interact with the catalytic site of the Ca²⁺-ATPase. This site undergoes a hydrophilic-hydrophobic transition during the catalytic cycle (42-48). In the hydrophilic state, the ATPase is phosphorylated by ATP and in the hydrophobic state it is phosphorylated by P₃. The correlation found between the hydrophobicity and the inhibitory activity of the anesthetics (Table I and Fig. 4) indicates that hydrophobic interactions are involved in their competition with P₃ and Mg²⁺. Benzocaine did not compete with Mg²⁺ (Fig. 3B), suggesting a requirement for a positively charged lateral amino group for electrostatic interaction with...
the Mg$^{2+}$-binding site on the enzyme. The differential effects of benzocaine on the one hand and the positively charged anesthetics on the other support the proposal that Mg$^{2+}$ and P, bind to different sites (9, 36), with the two sites being closely related in the hydrophobic environment of the catalytic site.

Data obtained in different laboratories suggest that Ca$^{2+}$ may be released from light sarcoplasmic reticulum vesicles through the Ca$^{2+}$-ATPase (8, 9, 49–54). The following findings indicate that the Ca$^{2+}$ efflux promoted by the anesthetics is mediated by the Ca$^{2+}$-ATPase. 1) Local anesthetics induce Ca$^{2+}$ efflux and decrease the phosphoenzyme level in the same concentration range (Table I). 2) The efflux is inhibited by monovalent, divalent, and polyvalent cations in the same concentration range as that required for interaction of the cations with the pump (Figs. 5–10). 3) Binding of ATP or ADP to the enzyme blocks the Ca$^{2+}$ efflux promoted by the anesthetics (Figs. 10 and 11). These findings do not exclude the possibility that the anesthetics may modify the protein–lipid interface of the ATPase leading to a change in the ATPase properties (41). In this case, however, phosphorylation of the ATPase by P, and binding of substrate to the enzyme should block the effect of the anesthetics on the lipids which bind to the enzyme.

The cations tested in this study are likely to interact either with the high affinity Ca$^{2+}$ sites of the enzyme or with specific cation-binding regions in the catalytic site. An interesting feature of the organic polyamines used is that their effectiveness in blocking the action of anesthetics on Ca$^{2+}$ efflux depends on the number of positive amino groups and on the size of the molecule used. In addition, the concentration range in which polyamines block Ca$^{2+}$ efflux in the light fraction is the same as that observed in junctional vesicles (6, 7, 55), indicating that they are not specific ligands of Ca$^{2+}$ release channels found in the junctional vesicles.

At present we do not know the mechanism by which heparin potentiates the effect of the positively charged anesthetics (Fig. 12). Recently it has been shown that heparin inhibits the Ca$^{2+}$ efflux promoted by inositol 1,4,5-trisphosphate in animal (56) and plant membranes (57). Finally, the finding that K$^+$, at concentrations that are found intracellularly, abolishes the effect of the local anesthetics on the rate of Ca$^{2+}$ efflux (Fig. 8) suggest that these compounds are not able to enhance the Ca$^{2+}$ efflux of intracellular compartments such as the sarcoplasmic reticulum. It is known that the local anesthetics inhibit the Ca$^{2+}$ transport ATPase of synaptosomal plasma membrane (58, 59). However, we do not know at present whether or not these anesthetics are also able to enhance the efflux of Ca$^{2+}$ through the Ca$^{2+}$-ATPase found on the plasma membrane of nerve cells.

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