MOLECULAR PHARMACOLOGY, 17, 61–65

The Modification of Unidirectional Calcium Fluxes by Dibucaine in Sarcoplasmic Reticulum Vesicles from Rabbit Fast Skeletal Muscle

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Received June 19, 1979; Accepted August 30, 1979

SUMMARY

NASH-ADLER, P., C. F. LOUIS, G. FUDYMA AND A. M. KATZ. The modification of unidirectional calcium fluxes by dibucaine in sarcoplasmic reticulum vesicles from rabbit fast skeletal muscle. *Mol. Pharmacol.* 17: 61–65 (1980).

The ability of dibucaine to modify unidirectional calcium fluxes was studied in sarcoplasmic reticulum vesicles prepared from rabbit fast skeletal muscle. Dibucaine inhibited initial calcium uptake velocity in a concentration-dependent manner; at 7.4 μM Ca^{2+}, approximately 0.8 mM dibucaine was required to achieve half-maximal inhibition of the calcium pump. This inhibitory effect of dibucaine was increased when external Ca^{2+} was decreased. Lower dibucaine concentrations (0.08–0.64 mM), however, stimulated calcium uptake when added to calcium-filled vesicles. This stimulatory effect of dibucaine was due to a decrease in the calcium efflux rate of the vesicles. Higher dibucaine concentrations (>0.9 mM) increased calcium efflux and inhibited calcium influx when added to calcium-filled vesicles. These complex effects of dibucaine on calcium transport by the sarcoplasmic reticulum may reflect the ability of this drug to stabilize the membrane at lower concentrations and to disrupt or induce lysis of the membrane at higher concentrations.

INTRODUCTION

Local anesthetic agents such as tetracaine, dibucaine, procaine, and lidocaine have been shown to inhibit calcium uptake[1] (1–4) and Ca^{2+}-activated ATPase activity (3, 5) of sarcoplasmic reticulum vesicles. Because inhibition of calcium uptake occurs at lower anesthetic drug concentrations than does inhibition of ATPase activity, Martonosi *et al.* (3) suggested that these inhibitory effects may not be due entirely to inhibition of the ATP-dependent calcium pump present in the membrane of the sarcoplasmic reticulum. An additional effect to increase calcium permeability was suggested also to play a role in the inhibition of calcium uptake by local anesthetic agents (3, 5, 6). Such an increase in calcium permeability would be expected to slow net calcium uptake by increasing calcium efflux from the sarcoplasmic reticulum vesicles. Although local anesthetic agents have been found to promote spontaneous calcium release from the sarcoplasmic reticulum at concentrations that inhibit initial calcium uptake, these drugs have also been reported to block ADP-dependent calcium release (5, 6, 9) and caffeine-induced calcium release (8) from sarcoplasmic reticulum vesicles.

In an attempt to clarify the modification of calcium release by local anesthetic agents, the effects of dibucaine on unidirectional calcium fluxes in rabbit skeletal muscle sarcoplasmic reticulum vesicles were investigated.

MATERIALS AND METHODS

Preparation of sarcoplasmic reticulum vesicles from rabbit fast skeletal muscle and measurements of calcium uptake and calcium release by Millipore filtration technique were described previously (10, 11). Ionized calcium concentrations outside the vesicles (Ca_o)[2] were calculated according to the equations of Katz *et al.* (10) except that, when CaEGTA buffers were used, a binding constant for $CaEGTA^{-2}$ of 10^{11} was used.

Calcium influx was measured by addition of tracer amounts of high specific activity carrier-free $^{45}CaCl_2$ to a reaction mixture in which $^{40}CaCl_2$ was used for the initial calcium uptake reaction. A duplicate reaction was started with $^{45}CaCl_2$ instead of $^{40}CaCl_2$ to permit assessment of net calcium uptake and release prior to and after addition of tracer calcium, as well as to allow calculation of total calcium concentration outside the vesicles at the time of tracer addition.

Supported by NIH Grants HL 21812 and HL 22135 and grants from the American Heart Association and the University of Connecticut Research Foundation. C. F. Louis is a MDA Research Fellow.

[1] As used in this article, "calcium uptake" and "calcium release" refer to the rates of net gain or loss of calcium by the vesicles, "calcium influx" and "calcium efflux" refer to rates of unidirectional calcium fluxes into and out of the vesicles, respectively.

[2] The abbreviations used are: Ca_o, ionized Ca^{2+} concentration outside the vesicles; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N*, *N'*-tetraacetic acid.

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Calcium influx rates were calculated from the rate of tracer uptake, adjusted to reflect the average calcium concentration outside the vesicles during each time interval and expressed as nanomoles of influx per mg of protein. Calcium efflux was calculated by subtracting the rate of calcium uptake or release from the calcium influx rate (11).

Low buoyant density sarcoplasmic reticulum fractions ("light" vesicles), purified on a 20 to 60% (w/v) linear sucrose density gradient (12, 13), were used in all experiments. Unless otherwise stated, experiments were carried out at 25° in 120 mM KCl, 40 mM histidine buffer (pH 6.8), with 50 mM Tris–phosphate as the calcium-precipitating anion, 5 mM MgATP, and 6 μg/ml light sarcoplasmic reticulum vesicles. An ATP-regenerating system (0.15 mg/ml pyruvate kinase and 5 mM phosphoenolpyruvate) was used in most experiments to maintain ATP concentrations and to minimize ADP accumulation.

All reagents used were reagent grade, and deionized water was distilled from glass prior to use. Disodium ATP (Boehringer–Mannheim) was desalted and neutralized with Tris and $MgCl_2$ as described previously (10). Pyruvate kinase (Type III) was purchased from Sigma Chemical Company. Dibucaine HCl and phosphoenolpyruvate were obtained from K & K Laboratories and Boehringer–Mannheim, respectively.

All data are representative of series of at least three replicate experiments.

RESULTS

Effects on initial calcium uptake velocity. In accord with previous studies (4, 5), dibucaine inhibited calcium uptake. The dibucaine concentration required for half-maximal inhibition of initial calcium uptake velocity (IC_{50}) was approximately 0.5 mM (Fig. 1) when Ca_0 was 0.4 μM. When Ca_0 was increased to 7.4 μM, the IC_{50} for dibucaine was approximately 0.8 mM (Fig. 1), which was significantly higher than the IC_{50} at 0.4 μM Ca_0 ($p < 0.02$). Lineweaver–Burk plots such as that shown in Fig. 2 indicate that dibucaine increased the Ca_0 needed to achieve half-maximal calcium uptake velocity without

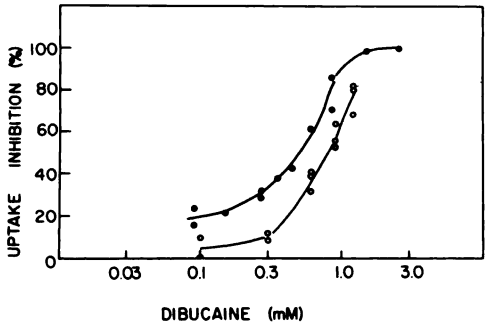

FIG. 1. *Effect of dibucaine on initial calcium uptake velocity*
Initial calcium uptake was determined in reaction mixtures containing 120 mM KCl, 40 mM histidine buffer (pH 6.8), 25–42 μM $^{45}CaCl_2$, 5 mM MgATP, and 50 mM Tris phosphate as calcium-precipitating anion. Initial Ca_0 was either maintained at 0.4 μM with the use of a CaEGTA buffer (●) or was maintained at 7.4 μM (○). All reactions were started by the addition of "light" vesicles (6 μg/ml) at zero time. Dibucaine concentrations ranged from 0.09 to 2.55 mM.

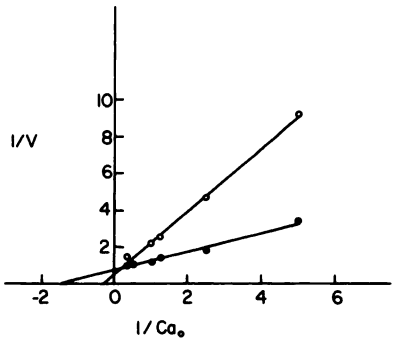

FIG. 2. *Lineweaver–Burke Plot*
CaEGTA buffers were used to maintain initial Ca_0 in the range 1×10^{-7} to 3×10^{-6} M. All reactions were started with light vesicles (6 μg/ml) at zero time. Total $CaCl_2$ was 25 μM; the dibucaine concentration was 0.5 mM. Control and dibucaine-containing mixtures were run simultaneously. Control (●), dibucaine (○).

significantly affecting the maximal velocity of the reaction.

Experiments in which the vesicles were preincubated for various times with dibucaine demonstrated a time-dependent increase in the inhibitory effect of a given concentration of the local anesthetic agent. In one experiment, for example, prolonging the preincubation time from 0 to 30 min increased the percentage inhibition of initial calcium uptake velocity by 0.75 mM dibucaine from 27 to 69%.

Effects on time-dependent changes in calcium content. Calcium uptake reactions, carried out under conditions where Ca_0 remains high throughout the reaction, characteristically exhibit four distinct phases: initial calcium uptake, an initial maximum of calcium content, spontaneous calcium release, and reuptake of calcium (14). When dibucaine was added at the start of the reaction, low concentrations that had little effect on initial calcium uptake velocity had complex effects on the subsequent time-dependent changes in calcium content (Fig. 3). A concentration of 0.25 mM dibucaine, which did not significantly inhibit initial calcium uptake velocity, prolonged the initial uptake phase and thereby increased the calcium capacity[3] of the vesicles; this dibucaine concentration also inhibited calcium reuptake. Calcium capacity was also increased by 0.5 mM dibucaine, which inhibited initial calcium uptake velocity and abolished calcium reuptake. A higher concentration of dibucaine (0.75 mM) inhibited initial calcium uptake velocity and abolished calcium reuptake. Dibucaine concentrations of 1.0 and 1.5 mM almost completely inhibited calcium uptake and greatly reduced the calcium capacity.

Effects on calcium-containing vesicles. To determine whether the effects of dibucaine on the time-dependent changes in calcium content reflected a slow onset of drug action, dibucaine was added to calcium-containing vesicles at various times during ongoing calcium uptake reactions. A concentration of dibucaine near the IC_{50} for initial calcium uptake (0.75 mM) also inhibited calcium uptake when added 2 min after initiation of the reaction

[3] The term calcium capacity is defined as the maximum calcium content attained by the vesicles at the initial maximum of calcium content prior to the phases of calcium release and reuptake.

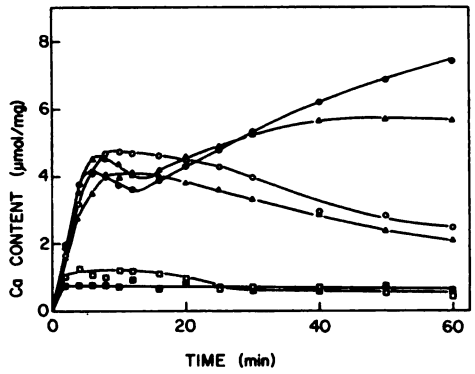

FIG. 3. *Effect of dibucaine at zero time on time-dependent changes in calcium content*

Reactions were started by addition of light vesicles (6 μg/ml) to reactions containing 0 (●), 0.25 mM (△), 0.5 mM (○), 0.75 mM (▲), 1.0 mM (□), or 1.5 mM (■) dibucaine.

(Fig. 4). However, addition of the same dibucaine concentration at the initial maximum of calcium content caused renewed uptake of calcium and thereby increased the calcium capacity of the vesicles. When 0.75 mM dibucaine was added later, during the spontaneous calcium release phase, calcium release was transiently halted. Addition of 0.75 mM dibucaine during the calcium reuptake phase caused a slight, transient acceleration of calcium uptake followed by the appearance of calcium release. Thus, dibucaine could cause either an immediate increase or decrease in calcium content, depending on when the drug was added.

The ability of dibucaine to increase calcium capacity when added at the initial maximum of calcium content (Fig. 4) was examined further to define the concentration dependence of this effect. Addition of low concentrations of dibucaine (0.08–0.64 mM) at the initial maximum of calcium content caused renewed calcium uptake and thus increased calcium capacity, whereas a higher dibucaine concentration (1.3 mM) caused an increase in the rate of calcium release (Fig. 5). Significant increases in calcium content ($p < 0.02$) were seen after addition of 0.08 mM to 0.64 mM dibucaine, the maximum increase being observed after 0.64 mM dibucaine was added at the maximum of calcium content. When the data shown in Fig. 5 were normalized by defining the maximal increase in calcium content after dibucaine addition as 100%, ap-

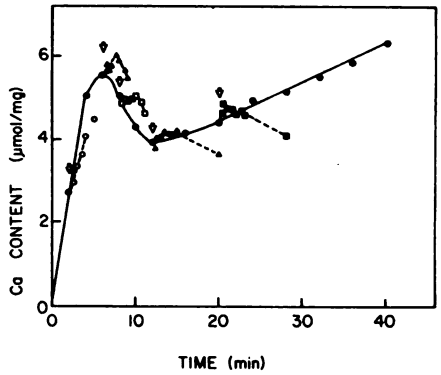

FIG. 4. *Effect of dibucaine added at various times to an ongoing calcium reaction* (●)

Dibucaine (0.75 mM) was added at 2 min (○), 6 min (△), 8 min (□), 12 min (▲), or 20 min (■) after the start of the reaction containing 6 μg/ml sarcoplasmic reticulum vesicles, 72 μM $CaCl_2$, 5 mM MgATP, an ATP-regenerating system, 50 mM Tris–phosphate, 120 mM KCl, 40 mM histidine buffer (pH 6.8). Portions of the control reaction were transferred into dibucaine at the above times. The amount of dibucaine was not large enough to induce any significant volume change.

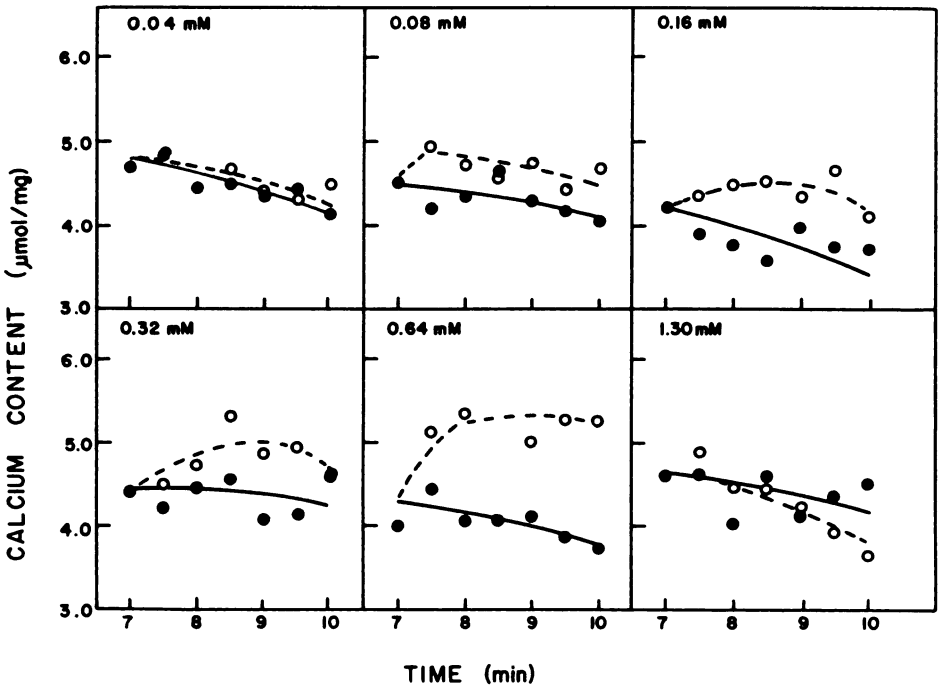

FIG. 5. *Effect of dibucaine added at the initial maximum of calcium content*

Portions of the control (●) reaction mixture were transferred into various concentrations of dibucaine (0.04–1.3 mM) at 7 min (○). Reaction mixtures were the same as those for Fig. 4.

proximately 0.13 mM dibucaine was required to produce a 50% increase in calcium content (Fig. 6). This dibucaine concentration is one-sixth the concentration needed to cause 50% inhibition of initial calcium uptake velocity (0.80 mM; Fig. 6).

Effects on unidirectional calcium fluxes. A series of calcium influx and calcium efflux measurements was made to determine whether the ability of dibucaine to increase calcium content (Fig. 5) was due to inhibition of calcium efflux, stimulation of calcium influx, or both. Addition of low concentrations of dibucaine (0.08–0.64 mM) at the initial maximum of calcium content, which induced significant increases in calcium content, had little effect on calcium influx (Fig. 7). Because these findings exclude the possibility that the increased calcium content was due to stimulation of calcium influx, it can be concluded that calcium efflux is selectively inhibited by these low dibucaine concentrations.

Higher dibucaine concentrations inhibit calcium influx when added at the initial maximum of calcium content (Fig. 7). The IC_{50} of calcium influx by dibucaine added at the initial maximum of calcium content was approximately 1.0 mM, which is similar to that which inhibited initial calcium uptake velocity half-maximally (Fig. 6). Calcium influx was completely inhibited by 1.8 mM dibucaine, a concentration which increased calcium efflux rate from 1.1 μmol/mg/min to 1.9 μmol/mg/min. Thus, the ability of these higher dibucaine concentrations to accelerate calcium release is due to both inhibition of calcium influx via the calcium pump and stimulation of calcium efflux across the membrane.

DISCUSSION

The range of dibucaine concentrations that inhibited initial calcium uptake velocity in sarcoplasmic reticulum vesicles (Fig. 1) is similar to that reported previously (1–3, 5). This inhibitory effect of dibucaine decreased as initial Ca^{2+} concentration was increased (Figs. 1 and 2). The finding that the inhibitory effect of dibucaine resembles kinetically a competitive inhibition with Ca^{2+} (Fig. 2) is in accord with previous studies (4).

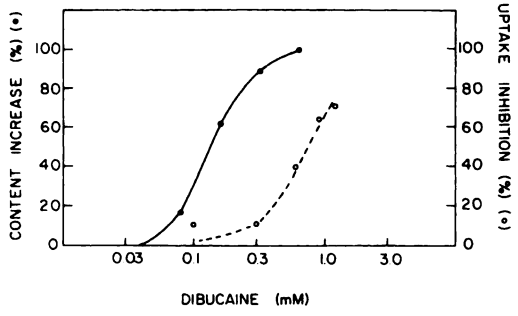

FIG. 6. *Concentration-dependent effect of dibucaine on calcium capacity at the initial maximum and inhibition of initial calcium uptake velocity*

Normalized data for increase in calcium content at the initial maximum were obtained from the experiment shown in Fig. 6 (●) where Ca_0 at 7 min was 7.4 μM. Percentage inhibition of initial calcium uptake velocity (○) by dibucaine was measured when vesicles were added at zero time to a complete reaction mixture containing dibucaine and an initial Ca_0 of 7.4 μM.

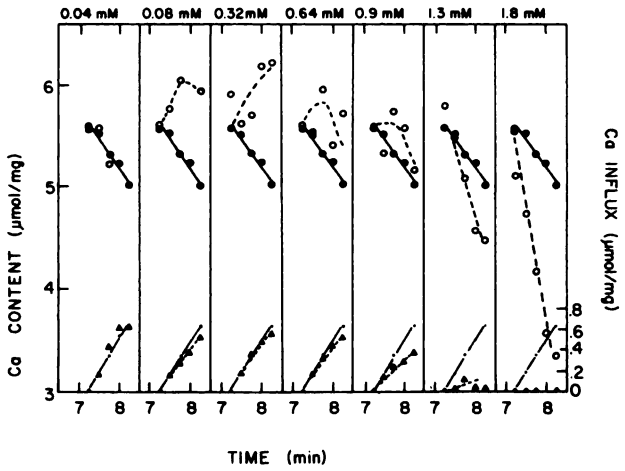

FIG. 7. *The effect of dibucaine on calcium content and calcium influx at the initial maximum in calcium content*

Calcium content (●, ○, upper curves) was measured in reactions containing 48 μM $^{45}CaCl_2$, portions of which were transferred into dibucaine at 7 min. Calcium influx reactions (●, △, lower curves) were measured in identical reaction mixture as described in METHODS in the presence (△) or absence (●) of dibucaine added at 7 min. Calcium content and calcium influx measurements were taken every 15 sec for 2 min. Calcium content after addition of dibucaine (○– – –○); control calcium content (●——●). Control values for calcium content and influx represent the mean of seven experiments.

Although addition of dibucaine prior to the start of the calcium uptake reaction caused only inhibition of subsequent calcium uptake, dibucaine added after the start of similar calcium uptake reactions could cause either an immediate increase or decrease in calcium content (Fig. 4). The nature of the response to dibucaine depended both on the concentration of the drug and the time at which it was added. During the initial phase of calcium uptake, when calcium efflux rates are near zero (15), addition of 0.75 mM dibucaine inhibited calcium uptake (Fig. 4). However, this concentration of dibucaine, added later during the calcium uptake reaction, could stimulate calcium uptake and increase calcium capacity (Fig. 4). Addition of 0.75 mM dibucaine at the initial maximum of calcium content, when calcium influx and efflux rates are equal in control reactions (11), caused almost complete inhibition of calcium efflux but less than 50% inhibition of calcium influx. Thus, the ability of dibucaine to increase calcium content when added at the initial maximum of calcium content is explained by a greater sensitivity of calcium efflux than calcium influx to the inhibitory effects of the drug (Figs. 6 and 7).

Tetracaine and dibucaine have been reported to exert two entirely different effects on passive calcium efflux from the sarcoplasmic reticulum (5, 6). These drugs inhibit the calcium efflux that is mediated by complete reversal of the calcium transport ATPase reaction (5, 6). In contrast, calcium efflux measured in the absence of ADP, which cannot represent reversal of the calcium transport ATPase reaction, was stimulated slightly by these local anesthetic agents (5, 6). The present finding that dibucaine can inhibit calcium efflux from calcium-filled vesicles is similar to the effect on calcium pump reversal (5, 6, 9) and is in accord with our earlier conclu-

sion that this calcium efflux is mediated by the calcium transport ATPase (15). Low dibucaine and tetracaine concentrations have also been reported to inhibit calcium efflux from mitochondria (16).

This ability of local anesthetic agents to inhibit passive calcium efflux from the sarcoplasmic reticulum could be due to incorporation of these amphiphilic drugs into the lipid bilayer (17–21), interaction with Ca^{2+} binding sites on membrane phospholipids (22, 23), or to direct interaction with a calcium channel, possibly involving the calcium pump ATPase. Specific binding to an ion channel site (cf. (24)) appears unlikely to explain the inhibition of calcium efflux because a variety of other amphiphiles of widely varying structure also inhibit this calcium efflux. These agents include X537A (10), A23187 (25), gramicidin D, valinomycin and nigericin (26), *beta*-adrenergic blocking agents (27), and a number of free fatty acids (28). In the case of the ionophores, inhibition of calcium efflux does not appear to be due to modification of a transmembrane monovalent cation gradient (29).

Higher dibucaine concentrations accelerate calcium release from the vesicles, as has been reported previously by Suko *et al.* (5, 6) and Johnson and Inesi (8). This effect is due both to inhibition of the calcium pump and to increased calcium efflux from the vesicles (3, 5, 17; Figs. 5 and 7). The inhibition of the calcium pump is associated with inhibition of the Ca^{2+}-dependent ATP hydrolysis (3, 6) an effect that appears to be due to inhibited decomposition of a phosphorylated intermediate of the calcium pump ATPase (3). As several investigators have suggested that modification of a lipid annulus surrounding the calcium pump ATPase can inhibit phosphoprotein decomposition (30), the inhibitory effects of local anesthetic agents and other amphiphilic agents on calcium transport and phosphoprotein decomposition might be mediated by alteration of this putative lipid annulus.

Low concentrations of a variety of lipid-soluble, surface-active compounds have been reported to decrease cation permeability and to protect several cell types and subcellular organelles from osmotic, mechanical or acid lysis (17, 20). Higher concentrations of these substances, however, induce membrane lysis (17). The complex response of sarcoplasmic reticulum vesicles to local anesthetic agents shows a similar concentration dependence in that these agents inhibit passive calcium efflux at low concentrations and increase calcium efflux and inhibit the calcium pump at high concentrations.

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