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Antagonism of Calmodulin by Local Anesthetics

Inhibition of Calmodulin-Stimulated Calcium Transport of Erythrocyte Inside-Out Membrane Vesicles

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SUMMARY

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Transport of calcium across the erythrocyte membrane is regulated by a Ca^{2+}-activated, Mg^{2+}-dependent ATPase which is stimulated by calmodulin. The regulation of Ca^{2+} transport by calmodulin was studied in inside-out membrane vesicles (IO vesicles) prepared from erythrocytes treated with protease inhibitors and lysed in media containing ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid and EDTA. Ca^{2+} uptake by IO vesicles was absolutely dependent on ATP, and the basal rate of uptake was 14.3 ± 1.1 nmoles/mg of IO vesicle protein per minute at a saturating concentration of free Ca^{2+} in the medium. Calmodulin stimulated Ca^{2+} uptake by an average of 3.5-fold to a V_{max} of 49.4 ± 3.9 nmoles/mg of IO vesicle protein per minute. The $K_{0.5}$ for calmodulin was 4.9 ± 0.9 nM and the maximal initial rate of Ca^{2+} uptake was attained at about 64 nM calmodulin. In a medium containing 80 mM Na^+, calmodulin reduced the $K_{0.5}$ for Ca^{2+} from 8.9 ± 1.8 μM (n = 3) to 5.0 ± 0.3 μM, but in a medium in which all Na^+ was replaced by K^+, calmodulin decreased $K_{0.5}$ Ca^{2+} to 1.4 μM free Ca^{2+}. Certain local anesthetics and drugs with local anesthetic-like properties (e.g., dibucaine, tetracaine, QX572, proadifen, mepacrine, quinine, propranolol, phenacaine, monocaine, and procaine), as well as trifluoperazine, inhibited calmodulin-stimulated Ca^{2+} uptake into IO vesicles. Calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase activity was inhibited to a degree equivalent to the effect on calcium uptake. Basal Ca^{2+} uptake (minus calmodulin) was only weakly inhibited; e.g., dibucaine at 1.0 mM inhibited stimulation by calmodulin 96.9%, but reduced basal Ca^{2+} uptake by only 11.4%. The local anesthetics increased the $K_{0.5}$ for calmodulin and reduced V_{max} for Ca^{2+} uptake. The reduction of V_{max} by local anesthetics was dose-dependent: e.g., 35% reduction at 0.25 mM dibucaine and 65% reduction at 0.5 mM dibucaine. V_{max} could not be restored to control levels by the addition of an excess of either calmodulin or Ca^{2+}. The antagonism of calmodulin by local anesthetics appeared to have elements of both competitive and noncompetitive inhibition. The antagonism versus Ca^{2+} was uncompetitive. These results are in agreement with our previous findings that certain local anesthetics are antagonists of calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase and calmodulin-stimulated cyclic nucleotide phosphodiesterase. It is suggested that certain of the well-known inhibitory effects of local anesthetics on Ca^{2+}-dependent cellular functions may be due to inhibition of the role played by calmodulin in facilitating these Ca^{2+}-dependent processes.

INTRODUCTION

Transport of calcium across the erythrocyte membrane is regulated by a Ca^{2+}-activated, Mg^{2+}-dependent ATPase. The activity of this enzyme in the intact membranes (1–3) after detergent-solubilization (4) and after reconstitution into lipid vesicles (5, 6) is markedly stimulated by the calcium-dependent regulator protein calmodulin.

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Calcium transport by inside-out membrane vesicles from red cells (7, 8) is also enhanced by calmodulin. Many other Ca^{2+}-dependent enzymes involved in glycogenolysis, lipolysis, cyclic nucleotide metabolism, membrane phosphorylation, and smooth muscle contraction are also stimulated by calmodulin (9). Calcium binds to calmodulin to produce the active form of the protein which can then interact with appropriate recognition sites on the enzymes. Many cellular functions which require Ca^{2+} are therefore dependent upon, or modulated by, calmodulin (9). In this way, calmodulin serves as a necessary constituent for the intracellular expression of the second-messenger or regulator functions of Ca^{2+}. Several antipsychotic drugs, including phenothiazines, are antagonists of calmodulin-dependent reactions, presumably by virtue of their ability to form a ternary complex with calmodulin-Ca^{2+}, which is thereby rendered unable to activate enzymes (10). Phenothiazines inhibit several calmodulin-dependent cellular functions (11, 12) and have been shown to prevent stimulation of the (Ca^{2+} + Mg^{2+})-ATPase of the erythrocyte membrane by calmodulin (13, 14).

Recently we reported that several local anesthetics inhibited both calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase of the erythrocyte membrane and calmodulin-stimulated brain cyclic nucleotide phosphodiesterase at concentrations which had little or no effect on the basal activities of these enzymes (15, 16). This effect is particularly intriguing because these local anesthetics are inhibitors of many calcium-dependent cellular functions such as motility, exocytosis, smooth muscle contraction, calcium transport, and cytoskeletal organization (see ref. 15 for citations). In this paper, we report the effects of local anesthetics on calmodulin-stimulated calcium transport in human erythrocyte IO vesicles.[3] Our results provide additional confirmation that certain local anesthetics specifically antagonize the effects of calmodulin and suggest that many of their pharmacological actions which are directed against Ca^{2+}-dependent processes may be due to this newly discovered mechanism of action.

MATERIALS AND METHODS

Preparation of inside-out red cell membrane vesicles. IO vesicles were prepared by modifications of the method of Steck (17). Freshly obtained human red cells were washed three times with 150 mM NaCl, 10 mM imidazole-HCl, pH 7.4, to remove plasma and the buffy coat and then resuspended in the same buffer at an hematocrit of 50%. Our method for obtaining IO vesicles was modified from that of Steck (17) and that of Larsen and Vincenzi (8) by employing protease inhibitors and Ca^{2+} chelators to decrease the likelihood of proteolysis, protein cross-linking, and lipolysis. The washed cells were first incubated at room temperature for 1 hr in the buffer solution containing 0.1 mM each of phenylmethanesulfonyl fluoride, 1-chloro-3-tosylamido-7-amino-1-2 heptanone, L-1-tosylamido-2-phenylethyl chloromethyl ketone, EDTA, and EGTA, and then lysed in 14 volumes of 10 mM imidazole HCl, pH 7.4, plus 1 mM EDTA at 4°. The membranes were collected by centrifugation at 20,000 × *g* (15 min). The tubes were rotated to draw the ghosts away from the membranous button containing leukocyte proteases at the bottom of the tubes, which was then removed by aspiration (17). The red cell membranes were resuspended in 40 volumes of cold 5 mM sodium phosphate, pH 8.0, and washed once in the same buffer. The pellets after centrifugation were resuspended in 0.5 mM sodium phosphate, pH 8.0, incubated on ice for at least 30 min, and then sedimented at 30,000 × *g* for 30 min. The supernatant was discarded and the pellet was kept at 4° overnight. Each milliliter of pellet volume was then resuspended with 1.0 ml of cold 0.5 mM phosphate buffer, pH 8.0, by Vortex mixing. This suspension was then passed three or four times through a 27-gauge needle fitted to a 1-ml syringe. The membranes were centrifuged at 30,000 × *g* for 30 min and washed once in 10 mM glycylglycine buffer, pH 7.1, with 0.025 mM $MgCl_2$, and then washed a second time in 20 mM glycylglycine, pH 7.1, containing 0.05 mM $MgCl_2$ (8). The IO vesicles can be stored for 4 days at 2° in this buffer without significant loss of activity.

Calcium transport. Vesicles were diluted 5-fold in transport buffer to a total protein concentration of 0.2–0.3 mg/ml. The composition of the final reaction mixture was as follows: 80 mM NaCl, 15 mM KCl, 2 mM glycylglycine, 1 mM MgATP, 1 mM $MgCl_2$, 0.1 mM ouabain, 0.1 mM EGTA, 0.2 μCi of ^{45}calcium chloride per milliliter, 0.2 mM $CaCl_2$ (specific activity = 1.7 × 10^6 cpm per micromole of calcium), 18 mM histidine, and 18 mM imidazole, pH 6.9. Incubations were carried out in a water bath at 37° and the reactions were started by the addition of ATP. Racks containing the reaction tubes were shaken by hand initially and at 1-min intervals thereafter. The free calcium concentration, unless otherwise stated, was 76 μM. Aliquots (125 μl) of the membrane suspension in transport buffer were removed at various times and filtered through a 0.45-μm Millipore filter (HAWP024) and the vesicles were washed with 5 ml of the same buffer as above (ice-cold), but with 2 mM $MgCl_2$ and without $^{45}CaCl_2$ or ATP. The radioactivity on the washed filters was measured in a scintillation counter. Uptake was corrected for nonspecific binding of ^{45}Ca to the Millipore filters, which usually amounted to not more than 10% of the ^{45}Ca associated with the membranes at zero time under basal conditions. Initial rates of calcium uptake were calculated from the uptake over the first 5 min, during which time the uptake was linear (Fig. 1).

IO vesicles were not separated from right-side-out vesicles, since the isolation procedure utilizing a dextran gradient decreased calcium uptake as previously described (8). The content of sealed IO vesicles was determined by measuring acetylcholinesterase activity. The enzyme in IO vesicles is located on the inner membrane surface and is not accessible to the substrate, but this "latent" activity can be revealed by disruption of the vesicles with Triton X-100 (17). IO vesicle protein is then calculated as total membrane protein × ($AChE_T$−AChE/

[3] The abbreviations used are: IO vesicles, inside-out plasma membrane vesicles; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; AChe, acetylcholinesterase; SKF 525A, β-diethylaminoethyl-2,2-diphenyl-pentanoate; QX572, *N*,*N*-bis(phenylcarbamoylmethyl) dimethylammonium chloride.

$AChE_T$), where AChE and $AChE_T$ are the enzyme activities measured in the absence and presence of Triton X-100, respectively. The ratio of $AChE/AChE_T$ was 0.48–0.49 in three experiments. Calcium uptake is expressed per milligram of IO vesicle protein, rather than total protein.

In order to study the relationship between calcium uptake and free Ca^{2+} concentration in the medium, the latter was adjusted with EGTA. EGTA was kept constant at 0.1 mM, and total calcium varied from 0.06 to 0.23 mM. To calculate free Ca^{2+}, we used the apparent binding constant of EGTA for calcium (2×10^6 M^{-1}) determined by Schwarzenbach *et al.* (18) and verified by Owen (19), employing a calcium-selective electrode. The free Ca^{2+} values are also corrected for Ca^{2+} binding to ATP as a function of pH and Mg^{2+} concentration.

Calmodulin was purified from human erythrocyte lysates by preparative gel electrophoresis as previously described (15). The protein was electrophoretically pure on sodium dodecyl sulfate-polyacrylamide gels and was equal in specific activity to a standard obtained from Dr. D. M. Watterson, The Rockefeller University (New York, N. Y.). The protein content was assayed by the method of Lowry *et al.* (20). Membrane protein was determined after removal of glycylglycine by washing membranes four times with phosphate buffer.

Proadifen (SKF 525A) and trifluoperazine were gifts from Smith Kline & French Laboratories (Philadelphia, Pa.). The sources of the other local anesthetics used were as follows: dibucaine (ICN Pharmaceuticals, Inc., Plainview, N. Y.); phenacaine (Mann Research Laboratories, Inc., New York, N. Y.), mepacrine, quinine, and procaine (Sigma Chemical Company, St. Louis, Mo.); QX572 (Astra Pharmaceutical Products, Inc., Worcester, Mass.), propranolol (Ayerst Laboratories, New York, N. Y.).

RESULTS

The calcium pump of the erythrocyte membrane, which extrudes calcium from the cell, is activated by ATP and calmodulin at sites on the cytoplasmic face of the membrane. One way in which to study regulation of the pump is to employ resealed IO vesicles prepared from hemoglobulin-free unsealed ghosts so that the cytoplasmic surface of the membrane is accessible to constituents of the medium. In this way, the effect of added calmodulin on (Ca^{2+} + Mg^{2+})-ATPase and calcium uptake can be evaluated readily. The preparations we employed contained about 50% sealed IO vesicles, with the remainder being right-side-out vesicles or unsealed vesicles. Only sealed IO vesicles can accumulate Ca^{2+} in the presence of ATP and the content of these vesicles is determined from the acetylcholinesterase assay (see Materials and Methods). Calcium uptake by IO vesicles was completely dependent upon the presence of ATP either in the absence or presence of calmodulin (Fig. 1). Calcium uptake was measured with time, and the initial rate of uptake was calculated from measurements made over the first 5 min (Fig. 1, *inset*), during which time less than 10% of the ATP was hydrolyzed and less than 15% of the calcium was taken up.

In vesicles prepared by previous methods (7, 8), the stimulation of the initial rate (i.e., over the first 5 min) of

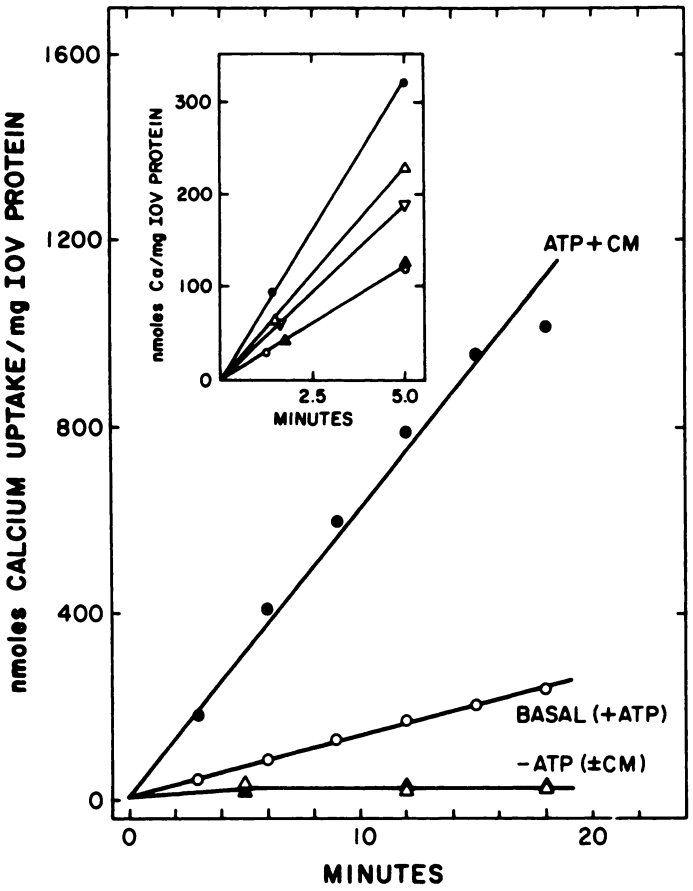

FIG. 1. *Effects of ATP and calmodulin (CM) on calcium uptake by IO erythrocyte membrane vesicles*

Calcium uptake was determined in the absence of ATP without CM (△), or with 64 nM CM (▲); in the presence of 1 mM ATP alone (○), or ATP plus 64 nM CM (●). Free Ca^{2+} was 76 μM. *Inset:* effect of dibucaine on the time course of calcium uptake by inside-out membrane vesicles (*IOV*). Basal uptake (○) and 64 nM CM-stimulated uptake (●) were measured in the absence of dibucaine, and CM-stimulated uptake was measured after the addition of 0.25 mM (△), 0.5 mM (▽), and 1.0 mM (▲) dibucaine. Free Ca^{2+} was 76 μM; ATP was 1 mM.

calcium uptake by an amount of calmodulin sufficient to produce a maximal effect (64 nM) was 2.36-fold (± 0.05 SEM). The maximal rate of uptake was 22.9 nmoles/mg of IO vesicle protein per minute at 37°. Treatment of washed red cells with EGTA, EDTA, and protease inhibitors (phenylmethanesulfonyl fluoride, 1-chloro-3-tosylamido-7-amino-1-2 heptanone, and L-1-tosylamido-2-phenylethyl chloromethyl ketone) prior to lysing in EDTA-buffer yielded IO vesicles that were stimulated 3- to 5-fold (average of 3.45-fold) by calmodulin and transported calcium at a maximal rate of 49.4 nmoles/mg of IO vesicle protein per minute at 37°. Half-maximal activation of calcium uptake was obtained with 5.9 ± 0.9 nM calmodulin and the maximal initial rate of uptake was attained at about 64 nM calmodulin (see below). As reported earlier by Larsen and Vincenzi (8), we observed that calcium previously taken up into the IO vesicles could be completely released by the ionophore A23187.

Local anesthetics added to the membrane vesicles prior to calmodulin suppressed the calmodulin-stimulated calcium uptake, whereas basal (minus calmodulin) uptake was relatively unaffected. Among the local anesthetics,

TABLE 1

Local anesthetic potency versus calmodulin-stimulated calcium uptake in erythrocyte membrane IO vesicles

The IC_{50} was estimated by varying the concentration of the drugs over a range of three to four values and calculating the concentration required for 50% inhibition of the initial rate of calmodulin-stimulated calcium uptake. The calmodulin and free Ca^{2+} concentrations were 64 nM and 76 μM, respectively.

Compound	IC_{50}
	*m*M
Proadifen	0.14
Dibucaine	0.27
Mepacrine	0.52
Propranolol	0.90
QX572	1.00
Quinine	1.40
Tetracaine	1.50
Phenacaine	3.0
Procaine	4.8
Monocaine	7.3

or drugs with local anesthetic-like properties, which inhibited calmodulin-stimulated calcium uptake were dibucaine, tetracaine, QX572, phenacaine, mepacrine, proadifen (SKF 525A), quinine, propranolol, monocaine, and procaine. The relative inhibitory potency of these drugs is shown in Table 1. A more detailed analysis of the effects of the local anesthetics is shown in Figs. 2–6. In these experiments, dibucaine was employed as the prototype of this class of drugs. Similar results were obtained with the other anesthetics. Figure 1 (*inset*) shows the initial time course of calcium uptake at several concentrations of dibucaine in the presence of a calmodulin concentration which produced a maximal rate of calcium uptake. The rate of calmodulin-stimulated calcium uptake was inhibited as a function of the dibucaine concentration. The uptake of calcium was completely suppressed to the basal level at a concentration of 1.0 mM dibucaine. The antagonism of calmodulin-activated calcium transport was accomplished with little or no effect on the basal transport of calcium; e.g., in seven experi-

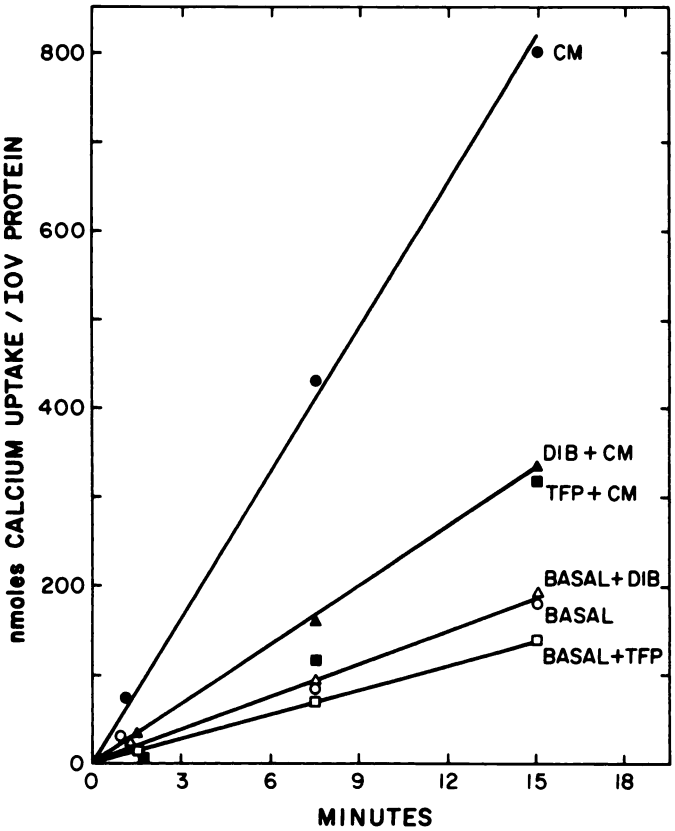

FIG. 2. *Effects of dibucaine (DIB) or trifluoperazine (TFP) on basal and calmodulin (CM) -stimulated calcium uptake into IO vesicles*

Basal uptake (○), basal uptake with 0.75 mM DIB (△), basal uptake with 75 μM TFP (□), uptake in the presence of 64 nM CM (●), uptake in the presence of CM plus 0.75 mM DIB (▲), or 75 μM TFP (■) Free Ca^{2+} was 76 μM; ATP was 1 mM.

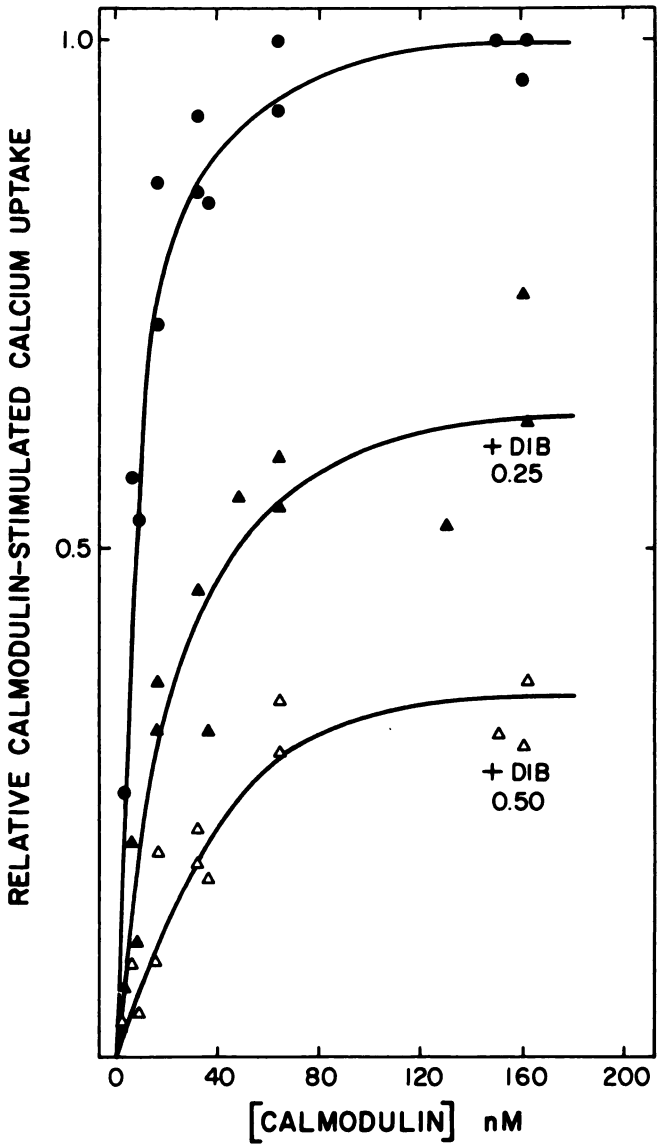

FIG. 3. *Effect of dibucaine (DIB) on the dose-response curve for calmodulin-stimulated calcium uptake*

Initial rate of calcium uptake was measured in three IO vesicle preparations as in Fig. 1 (*inset*): controls ●, plus 0.25 mM DIB ▲, plus 0.5 mM DIB △. The basal calcium uptake (minus calmodulin) has been subtracted, and all data are normalized to a value of 1.0 for the maximal uptake rate for each IO vesicle preparation. Free Ca^{2+} was 76 μM; ATP was 1 mM.

ments at 1.0 m-M dibucaine, calmodulin-activated calcium uptake was reduced 96.9 ± 3.7%, whereas basal uptake was inhibited by only 11.4 ± 2.0%. Trifluoperazine also inhibited calmodulin-stimulated calcium uptake and was about 10 times more potent than dibucaine on a molar basis (Fig. 2). Trifluoperazine at 50 μM also decreased basal calcium uptake by 10.8 ± 2.6%. Increasing the trifluoperazine concentration to 100 μM had only a small additional depressant effect on basal calcium transport, indicating that a limiting effect was approached. It is possible that the fraction of the basal calcium uptake which was inhibited by both trifluoperazine and local anesthetics was due to residual calmodulin not removed from the membranes during processing.

The inhibition of calmodulin-stimulated calcium uptake by local anesthetics was studied as a function of calmodulin concentration. These experiments (Figs. 3 and 4) indicate that dibucaine decreased V_{max} for calcium uptake. Increasing the calmodulin concentration could not overcome the effect of the local anesthetics on V_{max}. The maximal attainable rate of calcium uptake in the presence of excess calmodulin was reduced approximately 35 and 65% with 0.25 and 0.5 mM dibucaine, respectively. At lower concentrations of calmodulin, the degree of inhibition of calmodulin-stimulated calcium transport by the local anesthetics became progressively greater. The $K_{0.5}$ for calmodulin stimulation of calcium uptake was also increased by dibucaine. A double-reciprocal plot of representative data (Fig. 4) shows that the inhibition by dibucaine was of a "mixed-type," not purely competitive or purely noncompetitive.

Calcium uptake in IO vesicles was also measured as a function of free Ca^{2+} in the medium. Calmodulin increased not only the V_{max} for Ca^{2+} uptake but also decreased the $K_{0.5}$ for Ca^{2+}. The $K_{0.5}$ for Ca^{2+} in the absence of calmodulin was 8.9 ± 1.8 μM (n = 3) free Ca^{2+}, and 5.0 ± 0.3 μM (n = 3) in the presence of 65 nM calmodulin. These experiments were carried out with 80 mM Na^+ in the medium in order to make comparisons with our previous experiments on (Ca^{2+} + Mg^{2+})-ATPase activity in unsealed ghosts (15). When all Na^+ was replaced by K^+, the $K_{0.5}$ for Ca^{2+} was 8.7 μM free Ca^{2+}, which was decreased to 1.4 μM free Ca^{2+} in the presence of calmodulin. Schatzmann (21) found that an intracellular Ca^{2+} concentration of about 4 μM produced half-saturation of the erythrocyte calcium transport system. Figure 5 shows the effect of dibucaine on calcium uptake as a function of free Ca^{2+} concentration at 64 nM calmodulin. Dibucaine decreased V_{max}, and increasing the free Ca^{2+} concentration in the medium did not reverse the inhibitory effect of the local anesthetic. In the presence of 0.5 mM dibucaine, the maximal rate of calmodulin-stimulated calcium uptake was reduced by 65%. Higher concentrations of dibucaine further reduced the maximal attainable rate of calcium uptake. The basal rate of calcium uptake, as a function of free Ca^{2+} concentration, was unaffected by 0.5 mM dibucaine. Double-reciprocal plots of the calcium uptake as a function of free Ca^{2+} concentration in the medium showed a parallel shift of the lines by dibucaine, indicating that the inhibition was uncompetitive. We have previously shown (15) that local anesthetics decreased the V_{max} of (Ca^{2+} + Mg^{2+})-ATPase and that this effect could not be overcome by increasing the free calcium ion concentration.

DISCUSSION

In previous reports (15, 16) we showed that local anesthetics were antagonists of calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase of the erythrocyte membrane and calmodulin-stimulated brain cyclic nucleotide phosphodiesterase. At concentrations which inhibited the cal-

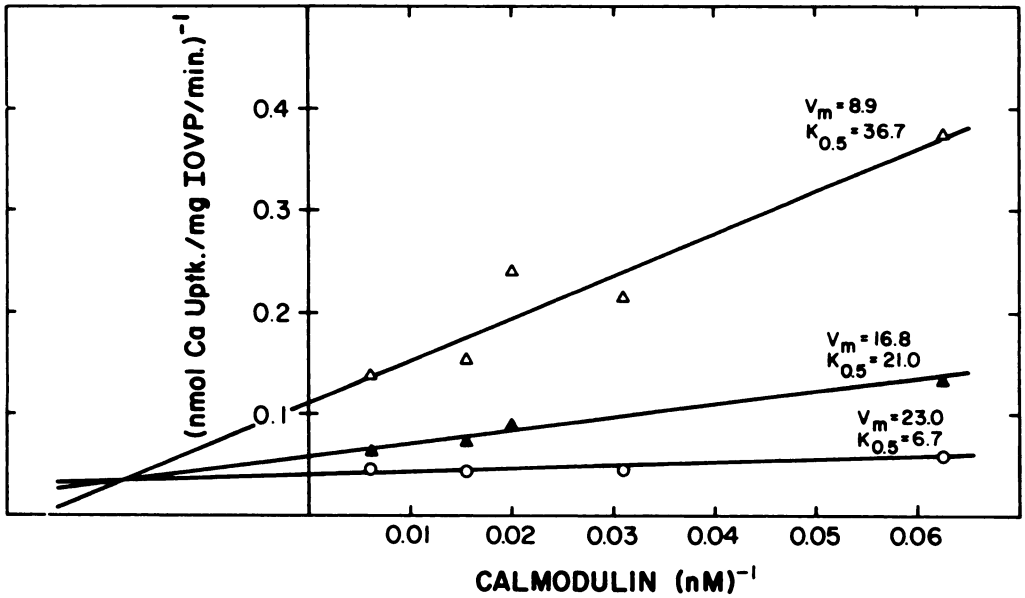

FIG. 4. *Double-reciprocal plot of an experiment in which initial rate of calcium uptake, as a function of calmodulin concentration, was measured in the absence of dibucaine* (○), *and in the presence of 0.25 mM* (▲) *or 0.5 mM* (△) *dibucaine*

Free Ca^{2+} was 76 μM. The *lines* were drawn according to the equation calculated by the method of least squares to fit the *data points*. V_m and $K_{0.5}$ for calmodulin were calculated from the equations. The basal rate of calcium uptake, 13 nmoles/min/mg of IO vesicle protein (IOVP), has been substracted; V_m represents the maximal rate of calmodulin-stimulated uptake.

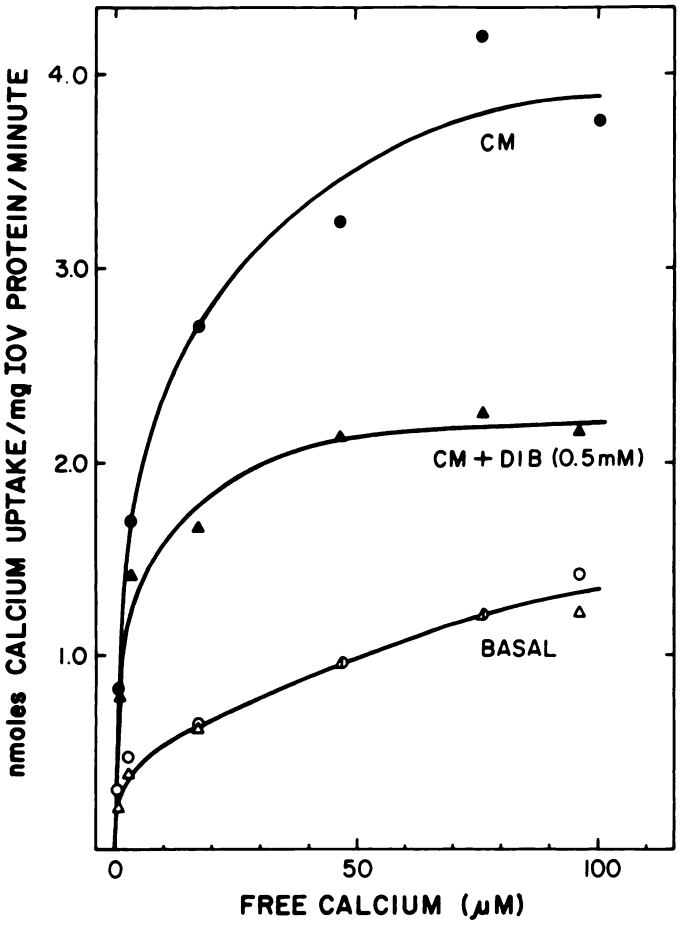

FIG. 5. *Calcium uptake as a function of free Ca^{2+} concentration in the medium*

Free Ca^{2+} concentration was adjusted by calcium-EGTA buffers (see Materials and Methods). The initial rate of calcium uptake was measured over a 5-min period as shown in Fig. 1 (*inset*): basal calcium uptake in the absence of calmodulin (*CM*) or dibucaine (*DIB*) (○), basal uptake in the presence of 0.5 mM DIB (△), uptake in the presence of 64 nM CM (●), and uptake in the presence of 0.5 mM DIB plus 64 nM CM (▲). ATP concentration was 1 mM. *IOV,* inside-out membrane vesicles.

modulin-stimulated activity of these enzymes by 90% or more, the local anesthetics had little or no effect on their basal activity. The experiments described in this report demonstrate further that ATP-dependent calmodulin-stimulated calcium uptake into erythrocyte membrane IO vesicles can also be inhibited by local anesthetics with little or no effect on basal calcium transport. The rate of calcium transport into IO membrane vesicles was reduced by local anesthetics as a function of drug concentration and to a degree comparable to the inhibition of ATPase activity (Fig. 6). Furthermore, the calmodulin-stimulated ATPase activity and the binding of ^{125}I-labeled calmodulin to erythrocyte membranes were previously shown to be inhibited by dibucaine to an equivalent degree over the same range of drug concentrations (15). These results taken together indicate that the ability of local anesthetics to inhibit calmodulin-dependent ATP-energized calcium transport in the red cell membrane is directly related to their ability to prevent the stimulation of the $(Ca^{2+} + Mg^{2+})$-ATPase by calmodulin. Unlike the ionophore A23187, the local anesthetics do not uncouple the two properties of the pump, i.e., calcium transport and ATP hydrolysis. The local anesthetics also act in a manner unlike ruthenium red, which inhibits both the basal and calmodulin-activated $(Ca^{2+} + Mg^{2+})$-ATPase of erythrocyte membrane to the same degree and may compete with Ca^{2+} for sites on the enzyme (14). We conclude from these observations that local anesthetics interfere with the binding of calmodulin to the calcium-transport ATPase and thereby simultaneously prevent both the stimulation of the ATPase activity of the enzyme and the enhancement of calcium transport.

Recently, anions were shown to stimulate Ca^{2+} transport in erythrocyte IO vesicles, presumably because the Ca^{2+} pump is electrogenic, causing the secondary accumulation of anions (22). Anion uptake was inhibited by reagents which react with Band 3 protein (the anion channel), and this in turn reduced Ca^{2+} uptake (22). Since local anesthetics can inhibit passive anion exchange in the red cell and in liposomes containing partially purified Band 3 protein (23), it is conceivable that they could affect Ca^{2+} transport indirectly by restricting anion transport. However, local anesthetics clearly have a direct effect on the enzymatic basis for the calcium pump, since they inhibit the stimulation of $(Ca^{2+} +$

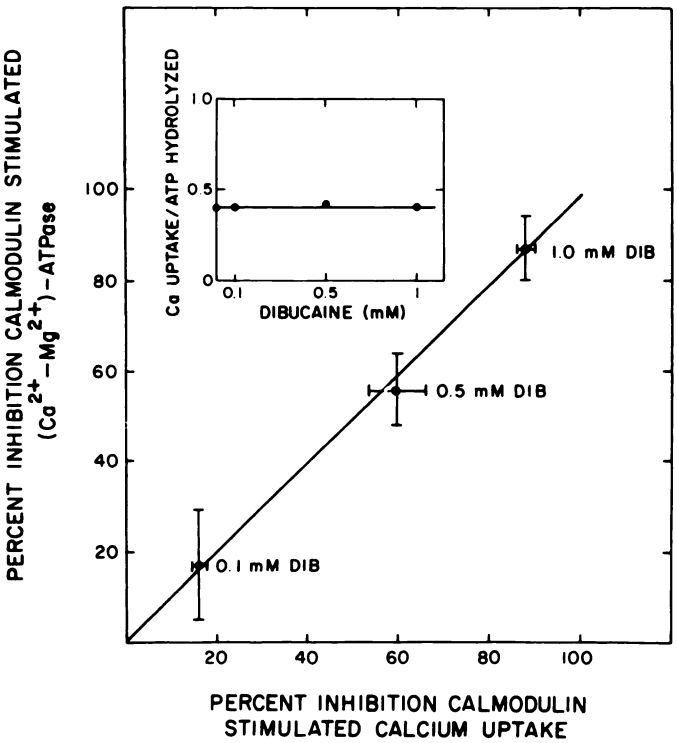

FIG. 6. *Effect of dibucaine (DIB) on calmodulin-dependent calcium uptake and $(Ca^{2+} + Mg^{2+})$-ATPase activity of IO vesicles*

The initial rate of calcium uptake was measured as in Fig. 1. In duplicate aliquots of the same vesicle preparation, $(Ca^{2+} + Mg^{2+})$-ATPase was measured over a 5-min period as previously described (15). Free Ca^{2+} was 76 μM and the calmodulin concentration was 64 nM. The basal calcium uptake and ATPase activities have been subtracted. The *inset* shows the molar ratio of calcium transported to ATP hydrolyzed. Waisman *et al.* (22) have reported that this ratio in IO vesicles varies with the anion composition of the medium from 0.25 with chloride to 0.87 with phosphate.

Mg^{2+})-ATPase by calmodulin in *unsealed* red cell ghosts (15) which cannot generate transmembrane gradients of either anions or cations (17).

We previously reported that local anesthetics not only shifted the calmodulin dose-response curve for ATPase activation to the right, thereby increasing the apparent $K_{0.5}$ for calmodulin, but the drugs also reduced the V_{max} of the enzyme (15). In the present study, a comparable effect on calcium transport has been observed. The apparent $K_{0.5}$ for calmodulin stimulation of the rate of calcium uptake was increased by the local anesthetics, and the V_{max} for calcium transport was decreased as the drug concentration was increased. Because neither the inhibition of ATPase activity nor the inhibition of calcium transport by local anesthetics was overcome by addition of excess calmodulin, a simple competitive antagonism between the modulator protein and local anesthetics cannot account entirely for the results observed with this enzyme. Local anesthetics also inhibited specific calmodulin-activated cyclic nucleotide phosphodiesterase, but in that case the inhibition was clearly competitive versus calmodulin (15, 16). Unlike the (Ca^{2+} + Mg^{2+})-ATPase, the cyclic nucleotide phosphodiesterase is a soluble enzyme. It is possible that the local anesthetics affect stimulation of membrane enzymes by calmodulin in a more complex way than they affect stimulation of soluble enzymes. The membrane ATPase appears to be inhibited in part by a competitive antagonism of calmodulin, but in addition by a second mechanism that is not reversible by either excess calmodulin or Ca^{2+}. This additional noncompetitive mechanism is unknown, but it may be due to interactions of local anesthetics with phospholipids (24). Phospholipids are required for erythrocyte (Ca^{2+} + Mg^{2+}) ATPase activity (4, 5, 25), and the enzyme can even be activated by certain detergents (4, 5) and acidic phospholipids (26) to a degree comparable with that produced by calmodulin. It is possible that local anesthetics affect the immediate lipid environment of the erythrocyte (Ca^{2+} + Mg^{2+})-ATPase, thereby altering its conformation and impairing the ability of calmodulin to stimulate the enzyme, perhaps by making the calmodulin binding site on the enzyme inaccessible.[4] Studies on detergent-solubilized enzyme or reconstituted enzyme in different lipid environments may help to resolve the molecular mechanisms of action of the anesthetics on the (Ca^{2+} + Mg^{2+})-ATPase. An important point to be addressed is whether local anesthetics would affect activation of the ATPase by acidic phospholipids.

Phenothiazines, such as trifluoperazine, also inhibit calmodulin-dependent stimulation of erythrocyte membrane (Ca^{2+} + Mg^{2+})-ATPase (13, 14). In this paper, we have shown that calmodulin-stimulated calcium transport is also inhibited by trifluoperazine. Phenothiazines appear to act as competitive inhibitors of calmodulin (10), although inhibition of (Ca^{2+} + Mg^{2+})-ATPase by trifluoperazine at a concentration of 100 μM may not be surmountable by an excess of calmodulin (13, 14). Trifluoperazine binds to high-affinity sites on calmodulin in the presence of Ca^{2+} (10), and the ternary complex of calcium-calmodulin-trifluoperazine apparently does not activate calmodulin-sensitive enzymes.[5] Although there appear to be some similarities between the effects of local anesthetics and trifluoperazine, the molecular mechanisms responsible for the effects of both types of inhibitors on (Ca^{2+} + Mg^{2+})-ATPase and calcium transport have yet to be fully elucidated.

The experiments reported in this paper demonstrate for the first time that local anesthetics can specifically antagonize a Ca^{2+}-dependent cellular function which is mediated by calmodulin. Calcium transport in sarcoplasmic reticulum (29) and Ca^{2+}-ATPase activity in brain cortex (30) can also be inhibited by local anesthetics, but the mechanisms involved are unknown; e.g., the rate of calcium uptake by sarcoplasmic reticulum vesicles from rabbit fast skeletal muscle was inhibited 50% by 0.8 mM dibucaine (29) and the brain Ca^{2+}-ATPase was inhibited 47% by 2.5 mM tetracaine at pH 7.8, but only 14% at pH 7.0 (30).

Local anesthetics have extraordinarily diverse pharmacological effects on cells, apart from nerve-blocking activity, and effects on both passive and active ion transport. Cytoskeletal organization, membrane structure, cellular motility, synaptic transmission, exocytosis, lipolysis, platelet aggregation, and muscle contraction can all be affected by local anesthetics (see ref. 15 for representative citations). Calcium is importantly involved in many of these processes and its actions in some cases may be mediated through the calcium-dependent regulator protein calmodulin (9). Although nerve-blocking activity due to effects on axon membrane Na^+ channels is probably unrelated to the calcium-dependent regulator protein, it is possible that some of the other pharmacological effects of local anesthetics noted above may be consequences of their antagonism of calmodulin-dependent enzymes. The drug concentrations which produce certain cellular effects are in many cases in the same range as the concentrations which antagonize the stimulation of the erythrocyte Ca^{2+} transport system (Table 1) or cyclic nucleotide phosphodiesterase (15, 16) by calmodulin. Much additional work on other calmodulin-dependent functions is necessary to define the significance of this particular aspect of the pharmacological actions of local anesthetics. Another point we must stress is that it is not known whether antagonism of calmodulin is a common feature of the action of all local anesthetics. Only a limited number of local anesthetic drugs have been utilized in our studies on Ca^{2+} transport, (Ca^{2+} + Mg^{2+})-ATPase, and calmodulin-activated cyclic nucleotide phosphodiesterase. Antagonism of calmodulin may be a pharmacological property of a subset of local anesthetics, and potency versus calmodulin may not necessarily correlate directly with the ability to block the action poten-

[4] Recently, local anesthetics (dibucaine and tetracaine) and chlorpromazine were shown to inhibit a Ca^{2+}-dependent protein kinase from rat brain cytosol which was stimulated by phospholipids and diglycerides (27). The anesthetics interfered with the activating effect of the phospholipid and did not affect the catalytic unit of the enzyme, which was fully active in the absence of Ca^{2+} and phospholipid. Dibucaine produced 50% inhibition at 0.5 mM.

[5] This may not be the only mechanism by which phenothiazines inhibit calmodulin-sensitive enzymes, since fluphenazine inhibited phospholipid-stimulated cyclic nucleotide phosphodiesterase in the absence of calmodulin (28).

tial in nerve fibers. Furthermore, the effectiveness with which local anesthetics antagonize various calmodulin-dependent functions in intact cells is likely to be dependent upon several factors, such as the relative calmodulin concentrations and distribution in different cells, the differing affinities of various enzymes for calmodulin, whether the calmodulin-dependent function involves cytoplasmic or membrane-bound enzymes, and, finally, whether calmodulin is a subunit of an enzyme (nondissociable) or binds reversibly to it only in the presence of Ca^{2+}. The rate and magnitude of changes in cytoplasmic free Ca^{2+} activity will determine both the rate of formation and the steady-state concentration of the Ca^{2+}-calmodulin complex and will thereby significantly influence the effects of a local anesthetic.

It is also evident that the effectiveness with which any particular local anesthetic inhibits different calmodulin-dependent reactions varies significantly; e.g., IC_{50} values are usually lower for the cyclic nucleotide phosphodiesterase (15) than for the (Ca^{2+} + Mg^{2+})-ATPase. The reason for these differences is not apparent as yet. Further work is required to determine whether the local anesthetics act on calmodulin itself, the calmodulin recognition sites on enzymes, or perhaps on both mechanisms in some cases.

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