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Interaction of Dibucaine and Calcium Ion on a Calcium Pump Reconstituted from Defined Components of Intestinal Brush Border

MASARU KUREBE

Department of Clinical Pharmacology, School of Pharmaceutical Sciences, Kitasato University, Tokyo 108, Japan

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SUMMARY

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The uptake of Ca^{++} by reconstituted proteoliposomes involves an ATP-independent as well as an ATP-dependent process. In the presence of 0.7 mM ATP, 1 mM dibucaine inhibited Ca^{++} uptake and (Mg^{++} + Ca^{++})-ATPase activity, but 0.2 mM dibucaine stimulated both. The latter reduced the velocity of ATP-independent Ca^{++} uptake at low Ca^{++} concentrations, but increased it at a high concentration (0.15 mM of Ca^{++}. ATP-dependent Ca^{++} uptake was facilitated by 0.2 mM dibucaine only in the presence of a high concentration of Ca^{++}. Kinetic analyses of the data obtained with the lower dose of dibucaine suggest that this compound competes with Ca^{++} for binding sites on the outer surface of membranes and that its interaction with Ca^{++} induces a conformational change of membranes accompanied by an increase in the passive permeability of Ca^{++} and activation of the ATP-mediated Ca^{++} pump.

INTRODUCTION

Local anesthetics lower the phase transition temperature of phospholipids (1), causing a tendency for lipids to arrange their long axes perpendicular to the plane of the film (2) and inducing a conformational change in protein molecules (3). They increase the fluidity of membrane lipids (4, 5) and natural membranes (6), and stabilize or labilize lysosomal membranes (7). A physicochemical study suggests that the increase in membrane disintegration rate by local anesthetics is the result of structural rearrangements with a weakening of lipid-protein interactions (8). It has been proposed that local anesthetics act at a hydrophobic site rather than a polar site in membranes (4). These physicochemical effects of local anesthetics on membranes may be related to their inhibitory effects on nerve action potentials (9), on active calcium transport of everted duodenum (10), and on the calcium pump of sarcoplasmic reticulum (11, 12).

Calcium ions, on the other hand, increase order in lipid multilayers (13), causing a segregation of phosphatidylserine in mixed bilayers of phospholipids (14, 15) and affecting the structural properties and permeability of cell membranes (16, 17). The antagonistic interactions of calcium and local anesthetics on membrane structure and function have been observed by many investigators (5, 18-21). It seems likely that calcium ions compete with local

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anesthetics for membrane binding sites (18, 20, 22). Local anesthetics inhibit the uptake of calcium by brain synaptosomes (23), sarcoplasmic reticulum (11, 12), and everted duodenum (10). In contrast, Narahashi *et al.* (24) have observed that the suppression of nerve conductance by local anesthetics is not affected by increased Ca^{++} concentrations. It is unsettled whether dibucaine and Ca^{++} interact with membrane sites to alter structural properties and calcium permeability or to induce conformational and functional changes of the calcium pump mediated by Ca^{++}-ATPase.

The present experiments were carried out, utilizing the technique of proteoliposome reconstitution from defined membrane components for a kinetic analysis of calcium transport, to investigate the action of dibucaine on ATP-independent and ATP-dependent calcium uptake in reconstituted ATPase proteoliposomes.

METHODS

Preparation of purified brush border. Male Wistar rats weighing approximately 250 g were used after overnight fasting. They were killed by decapitation. The mesenteric artery was perfused with ice-cold NaCl to remove blood. The small intestine was immediately isolated. The purified brush border was prepared from the mucosa by the method of Forstner *et al.* (25). All experiments were carried out with the purified brush border, which contained negligible amounts of DNA, succinate dehydrogenase, and NADPH–cytochrome *c* reductase. As judged from the results of marker enzyme and component assays, these purified brush border fractions were free from nuclear, mitochondrial, and microsomal contamination.

Preparation of defined membrane components from purified brush border. The procedure used for the preparation of the three fractions of purified brush border is summarized as a flow sheet in Fig. 1. The three fractions were prepared from a specimen of purified brush border (protein, 20 mg). Fraction 1, which contained a large amount of lipid, was prepared by extraction with chloroform–methanol (2:1) of a fraction of low density, which indicated a high specific activity of (Mg^{++} + Ca^{++})-ATPase. Fraction 2 was one of low density and contained deoxycholate-soluble (Mg^{++} + Ca^{++})-ATPase. Fraction 3 contained peripheral protein of the brush border, which was precipitated by divalent cations and solubilized with EDTA (26).

Reconstitution of (Mg^{++} + Ca^{++})-ATPase proteoliposomes. A mixture of fraction 3 (protein, 0.45 mg) and fraction 1 prepared from a fraction of low density (protein, 6.5 mg) was sonicated in 10 ml of 50 mM Tris-Cl buffer (pH 7) containing 1 mM $MgCl_2$, 0.2 mM sodium EDTA, and 4 mM sodium deoxycholate. To this suspension, 2 ml of fraction 2 (protein, 0.86 mg) was added. The mixture was dialyzed at 0–1° for 24 hr against 50 mM Tris-Cl buffer (pH 7) containing 1 mM $MgCl_2$ to remove deoxycholate and re-form vesicles, and then centrifuged at 100,000 × *g* for 60 min. The resulting pellet was resuspended in 50 mM Tris-Cl buffer (pH 7) containing 1 mM $MgCl_2$. After recentrifugation of this suspension, the resulting pellet was resuspended for use in Ca^{++} uptake and ATPase assays.

Calcium uptake assay. In measuring $^{45}Ca^{++}$ uptake, about 10 μg of protein of reconstituted proteoliposomes were added to a reaction medium containing 50 mM Tris-Cl (pH 7), 1 mM $MgCl_2$, and various concentrations of $^{45}CaCl_2$ in the presence and absence of 0.7 mM ATP. The final volume of the reaction mixture was 0.5 ml. After the reaction at 25°, 0.3 ml of the reaction mixture was filtered through a Sartorius membrane filter (pore size, 0.1 μm), followed immediately by washing with 1.5 ml of ice-cold 50 mM Tris-Cl (pH 7) containing 1 mM $MgCl_2$. The filtrate was free of proteoliposome protein. The filtered sediment was dissolved in Hyamine and counted with a liquid scintillation spectrometer. The radioactivity determined in the control assay was subtracted from that determined in the experimental sample. Protein was determined by the method of Lowry *et al.* (27) with bovine serum albumin as standard.

ATPase assay. ATP hydrolysis by reconstituted proteoliposomes was assayed un-

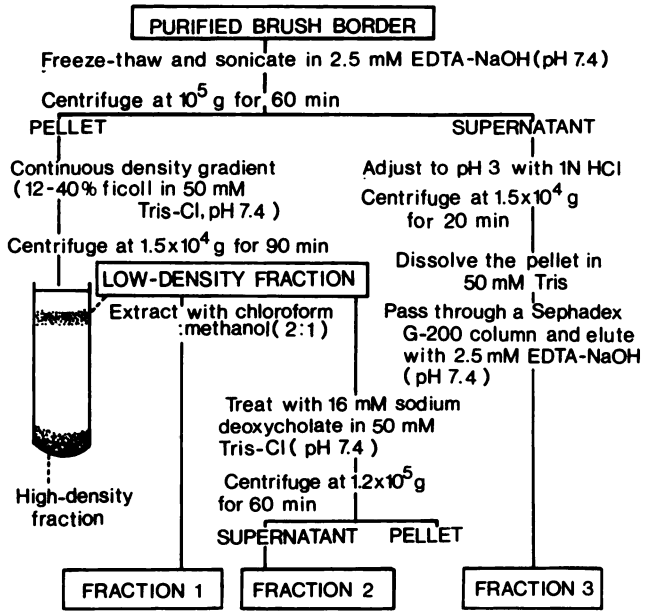

FIG. 1. *Procedure for preparation of defined membrane components from purified brush border*

The procedure for the preparation of fractions 1, 2, and 3 was started with a suspension of purified intestinal brush border (20 mg of protein) in 60 ml of 2.5 mM EDTA-NaOH (pH 7.4). In preparing fraction 1, a low-density fraction was extracted with chloroform-methanol (2:1). The extract was evaporated under nitrogen gas. After re-extraction of the residue with chloroform-methanol (2:1), fraction 1 was prepared by complete evaporation. Fraction 2 used in this experiment contained deoxycholate-soluble protein, from which a (Mg^{++} + Ca^{++})-ATPase protein was eluted as a single peak through a Sephadex G-200 column (2 × 90 cm) with a mixture of 2 mM sodium deoxycholate and 50 mM Tris-Cl (pH 7.4). From this peak, 92% of the deoxycholate-soluble protein was recovered as (Mg^{++} + Ca^{++})-ATPase. Fraction 3 was the first peak eluted from Sephadex G-200 gel (2 × 90 cm) filtration. All steps of the procedure were carried out at 0–4°.

der the same conditions in the reaction mixture used for Ca^{++} uptake experiments. After incubation of 0.5 ml of reaction mixture, containing proteoliposomes (about 10 μg of protein), 0.05 mM $CaCl_2$, 1 mM $MgCl_2$, 0.7 mM ATP and 50 mM Tris-Cl (pH 7), the reaction was stopped by adding 1 ml of ice-cold 10% trichloracetic acid. The released inorganic phosphate of the supernatant was analyzed (28).

RESULTS AND DISCUSSION

Time course of calcium uptake and ATP hydrolysis. The reconstituted proteoliposomes translocated Ca^{++} at a low rate, approximately 1.87 nmoles/mg of protein per minute, in the absence of ATP (Fig. 2). The rate of ATP-independent Ca^{++} uptake was approximately linear with time of incubation, except for an initial slight rise in Ca^{++} uptake. Addition of 0.7 mM ATP increased the velocity of Ca^{++} uptake to approximately 4.65 nmoles/mg of protein per minute. The rate of Ca^{++} uptake in the presence of ATP increased approximately linearly for at least 10 min and then decreased. Ca^{++} uptake in the presence of ATP was accompanied by ATP hydrolysis.

The proteoliposomes re-formed from (Mg^{++} + Ca^{++})-ATPase, lipids, and an EDTA-soluble protein of intestinal brush border could translocate Ca^{++} through ATP-independent and ATP-dependent processes. Ca^{++} uptake, however, was slower than the uptake of sarcoplasmic reticulum ATPase vesicles reconstituted with purified phospholipid and Ca^{++}-ATPase (29–31). Since ATP was added to the outside only, proteoliposomes were reformed to function as inside-out vesicles. These results suggest that a major pathway of Ca^{++} uptake in proteoliposomes is an ATP-mediated process via (Mg^{++} +

Ca^{++})-ATPase.

Effect of dibucaine on Ca^{++} uptake and ATPase activity. As shown in Table 1, 1 mM dibucaine inhibited (Mg^{++} + Ca^{++})-ATPase and Ca^{++} uptake in the presence of 0.7 mM ATP. This result is in harmony with the report of Suko *et al.* (32) that calcium uptake and Ca^{++}-dependent ATP hydrolysis were inhibited by local anesthetics in sarcoplasmic reticulum. At 0.2 mM, dibucaine stimulated (Mg^{++} + Ca^{++})-ATPase and Ca^{++} uptake in the presence of 0.7 mM ATP, but inhibited Ca^{++} uptake in the absence of ATP. This result suggests that at the low concentration of 0.2 mM dibucaine facilitates specific ATP-dependent Ca^{++} uptake, which was determined by subtracting ATP-independent uptake from uptake in the presence of ATP.

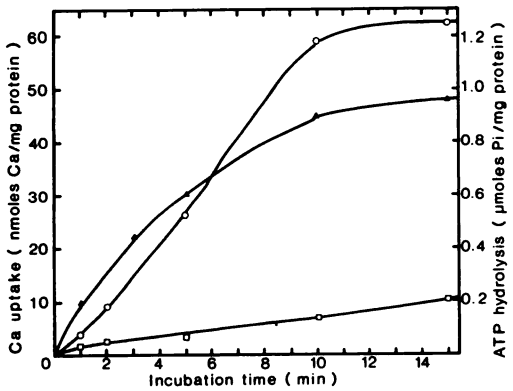

FIG. 2. *Time course of Ca^{++} uptake and ATP hydrolysis by reconstituted proteoliposomes*

The Ca^{++} uptake reaction was started by the addition of previously incubated proteoliposomes (approximately 10 μg of protein) to 50 mM Tris-Cl buffer (pH 7) containing 1 mM $MgCl_2$ and 0.05 mM $^{45}CaCl_2$ in the presence (○) and absence (□) of 0.7 mM ATP. The final volume of the reaction mixture was 0.5 ml. ATP hydrolysis (▲) was assayed under the same conditions in the reaction mixture used in the Ca^{++} uptake experiments, by determining the amount of P_i released from ATP.

Interaction of dibucaine and Ca^{++} on ATP-independent Ca^{++} uptake. The rate of Ca^{++} uptake in the absence of ATP increased with increasing concentrations of Ca^{++} (Fig. 3). The rate of ATP-independent Ca^{++} uptake rose markedly at Ca^{++} concentrations above 0.05 mM. Lineweaver-Burk plots of the rate of ATP-independent Ca^{++} uptake against Ca^{++} concentration up to 0.05 mM yielded straight lines (Fig. 4), indicating the presence of a saturable component in the process of ATP-independent Ca^{++} uptake. This may have been induced by ATP-independent Ca^{++} binding to a specific site. The double-reciprocal plots departed from linearity and became unsaturable at Ca^{++} concentrations above 0.05 mM (Fig. 4). This may be attributable to a Ca^{++}-induced conformational change in the membranes of closed proteoliposomes that produces a large increase in the passive diffusion of Ca^{++} rather than to the presence of bind-

TABLE 1

Effect of dibucaine on Ca^{++} uptake and ATPase activity in reconstituted proteoliposomes

Experimental details were the same as for in Fig. 2. Ca^{++} uptake and ATP hydrolysis were initiated by the addition of previously incubated proteoliposomes (approximately 10 μg of protein) to a medium (pH 7) containing 50 mM Tris-Cl, 0.05 mM $^{45}CaCl_2$, 1 mM $MgCl_2$, and various concentrations of dibucaine in the presence and absence of 0.7 mM ATP. The mixture (0.5 ml) was incubated at 25° for 1 min. Each value is the mean and standard deviation of 7 determinations.

Added dibucaine	(Mg^{++} + Ca^{++})-ATPase	Ca^{++} uptake	
		+ATP	−ATP
mM	*μmole P_i/mg protein/min*	*nmoles/mg protein/min*	
0	0.213 ± 0.017	4.65 ± 0.992	1.87 ± 0.276
0.1	0.254 ± 0.018[a]		
0.2	0.236 ± 0.018[b]	10.4 ± 2.40[c]	0.880 ± 0.185[c]
0.3	0.171 ± 0.025[a]		
0.4	0.132 ± 0.017[c]		
1.0	0.101 ± 0.015[c]	0.239 ± 0.067[c]	0.195 ± 0.059[c]

[a] $p < 0.01$.
[b] $p < 0.05$.
[c] $p < 0.001$.

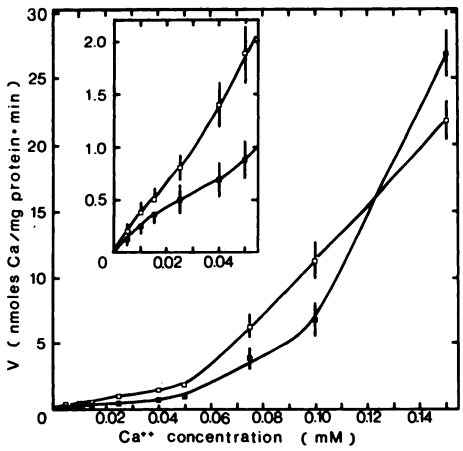

FIG. 3. *Effect of dibucaine on Ca^{++} uptake by reconstituted proteoliposomes at various Ca^{++} concentrations in the absence of ATP*

Experimental details were the same as for Fig. 2. The Ca^{++} uptake reaction was started by the addition of previously incubated proteoliposomes to a medium (pH 7) containing 50 mM Tris-Cl, 1 mM $MgCl_2$, and various concentrations of $^{45}CaCl_2$ in the presence (■) and absence (□) of 0.2 mM dibucaine. Each vertical bar represents a standard deviation.

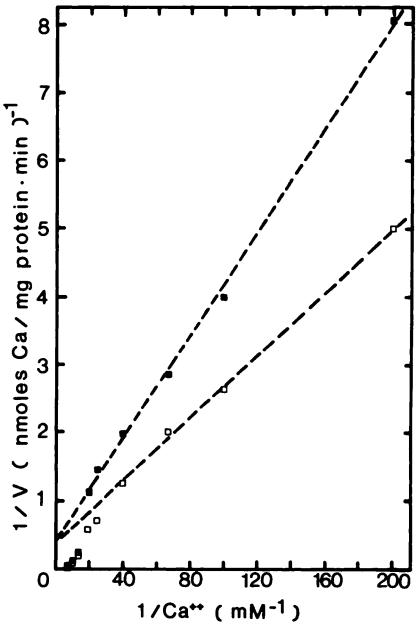

FIG. 4. *Interaction of dibucaine and Ca^{++} on Ca^{++} uptake by reconstituted proteoliposomes in the absence of ATP*

Double-reciprocal plots of the values given in Fig. 3. □, no dibucaine; ■, 0.2 mM dibucaine.

ing sites of different affinities in the process of ATP-independent Ca^{++} uptake. Many other studies (13–17) also suggest that calcium ions cause a conformational change of membranes. Ikemoto (30) assumed that the changes in Ca^{++} affinity might be related to changes in the structure of ATPase protein.

ATP-independent Ca^{++} uptake at Ca^{++} concentrations ranging from 0.01 to 0.1 mM was inhibited significantly ($p < 0.05$–0.001) by 0.2 mM dibucaine (Fig. 3). Double-reciprocal plots of the rate of ATP-independent Ca^{++} uptake in the presence and absence of 0.2 mM dibucaine yielded two straight lines with the same intercept at Ca^{++} concentrations up to 0.05 mM (Fig. 4), suggesting that calcium ions were competing with dibucaine. From these lines, the value of V_{max} was calculated to be 2.7 nmoles of Ca^{++} per milligram of protein per minute, with a K_m value of 0.058 mM and a K_i value of 0.30 mM. Most of the dibucaine molecules must be in the cationic form at pH 7. It is probable that calcium ions have about 5.3 times higher affinity than protonated dibucaine and compete with dibucaine for a negatively charged site on the outer surface of membranes only at low concentrations of Ca^{++} and dibucaine. At 0.2 mM, dibucaine increased the ATP-independent uptake of Ca^{++} significantly ($p < 0.001$) (Fig. 3). This effect of dibucaine may be a consequence of stimulation by Ca^{++}-induced conformational changes in the membranes of closed proteoliposomes. It has been reported that anesthetics enhance the fluidity of membrane lipids (5) and that dibucaine increases the calcium permeability of liposomes (33). Moreover, 1 mM lidocaine does not inhibit the activity of microsomal ATPase, but facilitates microsomal Ca^{++} uptake (34). Feinstein *et al.* (6) reported that the membrane fluidization inherent in the action of local anesthetics appears to be due to increased disorder in the lipid structure. The reports cited above lend support to the apparent mode of action of dibucaine indicated in this experiment.

Interaction of dibucaine and Ca^{++} on ATP-dependent Ca^{++} uptake. The rate of Ca^{++} uptake in the presence of ATP in-

creased with Ca^{++} concentration (Fig. 5), especially above 0.05 mM. The addition of 0.2 mM dibucaine facilitated this uptake significantly ($p < 0.001$) when the Ca^{++} concentration was more than 0.05 mM. ATP-dependent Ca^{++} uptake was calculated by subtracting ATP-independent Ca^{++} uptake (Fig. 3) from uptake in the presence of ATP (Fig. 5). Lineweaver-Burk plots of ATP-dependent Ca^{++} uptake yielded straight lines at Ca^{++} concentrations exceeding 0.05 mM in both the presence and absence of 0.2 mM dibucaine (Fig. 6). The presence of 0.2 mM dibucaine accelerated ATP-dependent Ca^{++} uptake relative to the activity of ATPase. These lines showed no positive intercept on the $1/v$ axis. The results shown in Fig. 6 and Table 1 suggest that 0.2 mM dibucaine induces a conformational change in the Ca^{++} pump in proteoliposomes with the cooperation of Ca^{++}, and that this change is responsible for the activation of (Mg^{++} + Ca^{++})-ATPase and the subsequent increase in ATP-dependent Ca^{++} uptake. This explanation, however, still does not account for the unsaturability of uptake indicated by the Lineweaver-Burk plots.

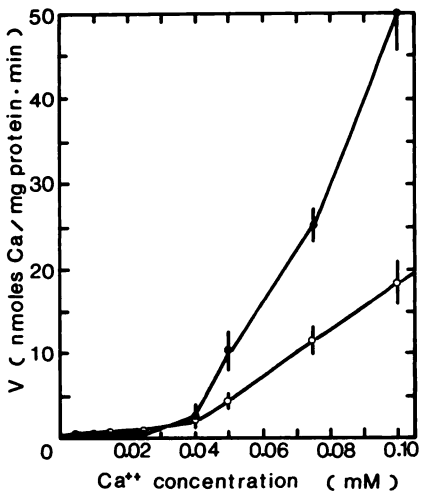

FIG. 5. *Effect of dibucaine on Ca^{++} uptake by reconstituted proteoliposomes at various Ca^{++} concentrations in the presence of ATP*

Experiments were carried out under the same conditions used for Fig. 2 to determine the effect of 0.2 mM dibucaine on Ca^{++} uptake in the presence of ATP. A 0.5-ml reaction mixture (pH 7), containing approximately 10 μg of proteoliposome protein, 50 mM Tris-Cl, 1 mM $MgCl_2$, 0.7 mM ATP, and various concentrations of $^{45}CaCl_2$, was incubated at 25° for 1-2 min in the presence (●) and absence (○) of 0.2 mM dibucaine. Each vertical bar represents a standard deviation.

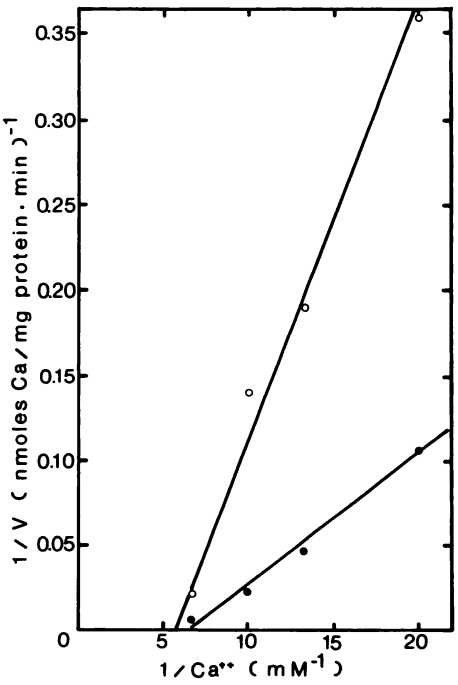

FIG. 6. *Interaction of dibucaine and Ca^{++} on ATP-dependent Ca^{++} uptake*

The reciprocal rate of ATP-dependent Ca^{++} uptake, calculated by subtracting ATP-dependent Ca^{++} uptake (Fig. 3) from Ca^{++} uptake in the presence of ATP (Fig. 5), is plotted against the reciprocal of Ca^{++} concentration. ○, no dibucaine; ●, 0.2 mM dibucaine.

A mechanism different from the effect of 0.2 mM dibucaine may have participated in the inhibitory effect of 1 mM dibucaine on (Mg^{++} + Ca^{++})-ATPase activity and Ca^{++} uptake (Table 1). It is generally accepted that an ATPase-lipid interaction is an important factor for the regulation of membrane-bound ATPase and Ca^{++} uptake. Dibucaine is thought to affect the interaction of ATPase and membrane lipid, which inhibits ATPase activity and Ca^{++} uptake. It is not yet clear, however, whether dibucaine affects Ca^{++} transport via (Mg^{++} + Ca^{++})-ATPase by a direct action on ATPase protein or by changing the protein-lipid interaction of the membranes. The present results suggest that the mechanism of dibucaine action on

Ca^{++} uptake in reconstituted proteoliposomes may consist of the following complicated factors: (a) competitive binding of low concentrations of Ca^{++} and dibucaine to the proteoliposome membrane; (b) a conformational change in membranes by a high concentration of Ca^{++} and a low concentration of dibucaine, which improves the accessibility of ATP and Ca^{++} to ATPase molecules, followed by activation of ATPase and an increase in ATP-mediated Ca^{++} uptake and in the passive permeability of Ca^{++}; and (c) inhibition of the lipid-ATPase interaction by a high concentration of dibucaine, which inactivates (Mg^{++} + Ca^{++})-ATPase and the Ca^{++} pump and also decreases ATP-independent Ca^{++} binding.

Further experiments are required to clarify the mechanism of interaction of Ca^{++} and dibucaine on the molecular conformation and Ca^{++} pump of reconstituted brush border membranes.

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