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SOME CHARACTERISTICS OF A Ca^{2+}-DEPENDENT ATPase ACTIVITY ASSOCIATED WITH A GROUP OF ERYTHROCYTE MEMBRANE PROTEINS WHICH FORM FIBRILS

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SUMMARY

A group of proteins with a fibrillar appearance and associated with ouabain-insensitive ATPase activity has been isolated from the human erythrocyte membrane. Some of the characteristics of a Ca^{2+}-dependent, Mg^{2+}-inhibited ATPase activity associated with this group of proteins are examined in this communication. The ATPase activity is not inhibited by ouabain. Mg^{2+} (0.5–6.0 mM) inhibited Ca^{2+} activation of ATP hydrolysis over the entire range of Ca^{2+} concentration tested (0–6 mM). GTP failed to serve as a substrate. A system of fibrils morphologically similar to the isolated fiber system can be seen on the erythrocyte membrane and has been identified in thin sections of ghosts. These fibrils were localized, at least in part, to the inner aspect of the unit membrane. A possible role for the Ca^{2+}-activated Mg^{2+}-inhibited ATPase and the associated fibrils in the maintenance of erythrocyte deformability is discussed.

INTRODUCTION

Erythrocyte membrane proteins have been extracted from native ghosts with diethyl ether[1], butanol[2], pyridine[3], ionic[4,5] and nonionic[6,7] detergents, salt solutions[8,9], and ATP *plus* mercaptoethanol[10]. Functional identification of proteins isolated by such procedures is complicated because either their location in the membrane is not known or their functional role, once isolated, is no longer demonstrable. The latter difficulty appears responsible, in many cases, for their designation as structural proteins.

About 20–25 % of human erythrocyte ghost protein can be solubilized by low ionic strength dialysis at room temperature in the presence of EDTA[26]. This protein fraction contains ouabain-insensitive ATPase activity and forms an elastic gel on the addition of greater than 1 mM divalent or 5 mM monovalent cation and appears as

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid.

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a network of interlacing fibrils when negatively stained and examined in the electron microscope. Concurrent with protein solubilization, the ghost fragments into spherical vesicles. In addition, the erythrocyte ghost loses ouabain-insensitive ATPase activity and a network of membrane fibrils. This ATPase activity consists of divalent cation sensitive and insensitive components which separate together with fibrillar appearing structures as part of the void volume on a Sepharose 4B column.

This communication describes some characteristics of a Ca^{2+}-dependent ATPase activity associated with this group of fibrillar proteins. In addition, electron microscope data is presented which shows that a network of morphologically similar fibrils is located on the inner surface of the ghost membrane. The Ca^{2+}-dependent ATPase described here may play a role in the maintenance of erythrocyte deformability.

MATERIALS AND METHODS

Human erythrocyte hemoglobin-poor ghosts were prepared by a modification[26] of the method of DODGE *et al.*[11]. Ghosts were diluted 1 to 4 with a solution of 0.5 mM EDTA, sodium salt at pH 7.4 and dialyzed at room temperature (21–23°) for 18 h against 200 vol. of the same EDTA solution containing 5 mg chloromycetin (Parke–Davis) per l to retard bacterial growth. The contents of the dialysis tubing were centrifuged at 40000 × *g* and separated into an insoluble residue consisting of vesicular ghost fragments and a protein rich supernatant. The supernatant solution was centrifuged again in a Spinco Model L ultracentrifuge for 1.5 h at 108000 × *g* at 4° and the small sedimentable residue which formed was discarded. Protein was determined by the method of LOWRY *et al.*[12]. The protein concentration of the supernatant varied between 0.4 and 0.5 mg/ml. Protein was then concentrated to about 4 mg/ml by vacuum dialysis at 4° against 50 mM glycine–Tris buffer (pH 7.4) or the EDTA dialysis solution. Detailed studies of the solubilization and identification of this protein are presented elsewhere[26].

[γ-^{32}P]ATP of known specific activity was prepared by the method of GLYNN AND CHAPPELL[13]. [^{32}P]Phosphate release from γ-labelled ATP in each 100-μl reaction volume (containing approx. 250 μg of soluble protein) was estimated after stopping the reaction with 3 vol. of cold 25% trichloroacetic acid. The precipitated protein was then centrifuged at 4° for 5 min at 2800 × *g*. A 300-μl aliquot of the clear trichloroacetic acid extract and 200 μl of 4% ammonium molybdate in 2 M H_2SO_4 were emulsified with 1.0 ml of isobutanol by vortex agitation. This suspension was centrifuged at 4° at 2800 × *g* for 3 min; the resulting upper organic phase contained [^{32}P]phosphomolybdate, and the lower, aqueous phase [^{32}P]ATP.

The quantity of [^{32}P]phosphomolybdate in a 0.5-ml aliquot of the isobutanol extract was determined in a Packard Model 3375 liquid-scintillation spectrometer using 15 ml of a counting fluid consisting of 200 ml of Triton X-100, 800 ml of toluene and 64 ml Liquifluor (Nuclear Chicago Corp.). An automatic internal standard was employed and a known sample of [^{32}P]ATP was included in each experiment. Alternatively, the release of P_i from ATP was determined colorimetrically by a modification of the method of BERENBLUM AND CHAIN[15]. The composition of the reaction mixture and the incubation conditions for determination of ATPase activity are given in the legends. ATPase activity was expressed routinely as nmoles $P_i \cdot$mg protein$^{-1} \cdot h^{-1}$.

For electron microscopy, aliquots of ghosts, vesicles, or protein were fixed with 4 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0) and spun at 108000 × *g* for 30 min. The pellet was post-fixed in 1 % OsO_4, dehydrated with ethanol and propylene oxide and embedded in Epon. Some pellets were enblock stained for 2 h with 0.5 % uranyl acetate prior to Epon embedding. Appropriate dilutions of ghosts, vesicles, and protein were also negatively stained with 1 % uranyl formate on formvar grids. Thin sections of the plastic embedded material and the negatively stained preparations were examined in a Philips EM 200 or EM 300 electron microscope.

RESULTS

Divalent cation-dependent ATP hydrolysis

The soluble protein extract possessed ATPase activity which was stimulated by divalent cations. Table I demonstrates that ATPase activity can be separated into a divalent cation sensitive and insensitive component. Ca^{2+} was the most effective cation for stimulating the hydrolysis of ATP. Of the other divalent cations tested only Mn^{2+} produced a significant stimulation. Sr^{2+}, Cd^{2+}, Zn^{2+} and Pb^{2+} either did not stimulate ATP hydrolysis or hydrolysis was not distinguishable from the activity in the absence of divalent cation. No ($Na^+ + K^+$)-stimulated, ouabain-sensitive ATPase activity was detectable in the supernatant fraction. Furthermore, ouabain (0.2 mM) did not inhibit or stimulate Ca^{2+}-dependent ATP hydrolysis.

TABLE I

EFFECT OF DIVALENT CATIONS ON STIMULATING ATP HYDROLYSIS

To a 100-μl reaction volume was added 2 mM of each divalent cation as the chloride, 1 mM Na_2ATP, 0.25 mM EDTA, and 25 mM HEPES buffer (pH 8.0). Incubation was at 37° for 60 min. Phosphate release was measured isotopically as described under MATERIALS AND METHODS. The specific activity of Ca^{2+}-dependent ATP hydrolysis was 12 nmoles $P_i \cdot mg\ protein^{-1} \cdot h^{-1}$. ATP hydrolysis is expressed as percent of the ATP hydrolysis with Ca^{2+}.

Divalent cation added	*Percent of Ca^{2+} activity*
Ca^{2+}	100
None	14
Mn^{2+}	67
Mg^{2+}	27

Some of the properties of the Ca^{2+}-dependent ATP hydrolysis by the supernatant protein were explored. Ca^{2+}-dependent ATP hydrolysis was linear over the 60-min incubation time (Fig. 1). The release of ^{32}P from ATP correlated with an increase in P_i in the reaction medium (Table II). In addition, the substrate specificity of the Ca^{2+}-dependent ATPase activity was demonstrated. GTP did not substitute for ATP as a substrate. At a Ca^{2+} concentration of 6.0 mM, ATP hydrolysis was maximal at about 1 mM ATP (Fig. 2). The half-maximal concentration for ATP under these conditions was approx. 0.1 mM.

The effect of increasing Ca^{2+} concentration on ATPase activity in the presence of 0.25 mM EDTA and 1.0 mM ATP is shown in Fig. 3. Note that 2 mM $MgCl_2$ inhibited Ca^{2+} activation while 2 mM $MgCl_2$ alone did not appreciably stimulate ATP hydro-

lysis. $MgCl_2$ at concentrations as low as 0.5 mM was an effective inhibitor of Ca^{2+}-dependent ATP hydrolysis at all concentrations of Ca^{2+} tested (0.5–0.6 mM).

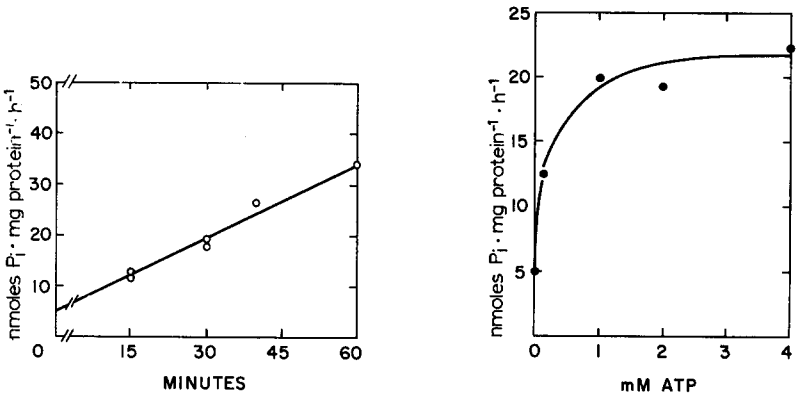

Fig. 1. The effect of incubation time on Ca^{2+}-dependent ATP hydrolysis. The reaction mixture contained 1 mM ATP, 2 mM $CaCl_2$, 1.25 mM EDTA, and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) buffer (pH 7.5). Incubation was at 37° for varying times.

Fig. 2. The effect of ATP concentration on Ca^{2+}-dependent ATP hydrolysis. To a reaction mixture of 100 µl were added 6 mM $CaCl_2$, 0.25 mM EDTA, and 25 mM HEPES buffer (pH 7.5) and varying concentration of Na_2ATP. Incubation was at 37° for 60 min.

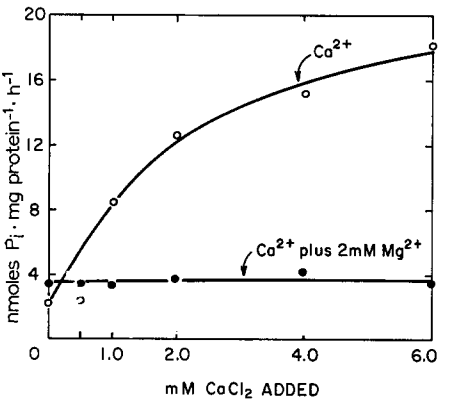

Fig. 3. Ca^{2+} activation (○) of ATP hydrolysis and Mg^{2+} inhibition of Ca^{2+} activation (●). The reaction mixture and incubation were the same as those in Fig. 3. The Mg^{2+} concentration, when present, was 2 mM.

Morphologic studies

When negatively stained and examined in the electron microscope, the supernatant was seen to contain a network of fibrils of varying length with a diameter of about 40–60 Å (Fig. 4A). These fibrils were detected only on the addition of those concentrations of monovalent cations or Ca^{2+} and Mg^{2+} which cause the supernatant to gel[26]. No membrane fragments were detected in these preparations. A similar system of fibrils was found on undialyzed erythrocyte ghost membranes when negative stains were performed in a manner identical to that used to demonstrate fibrils in

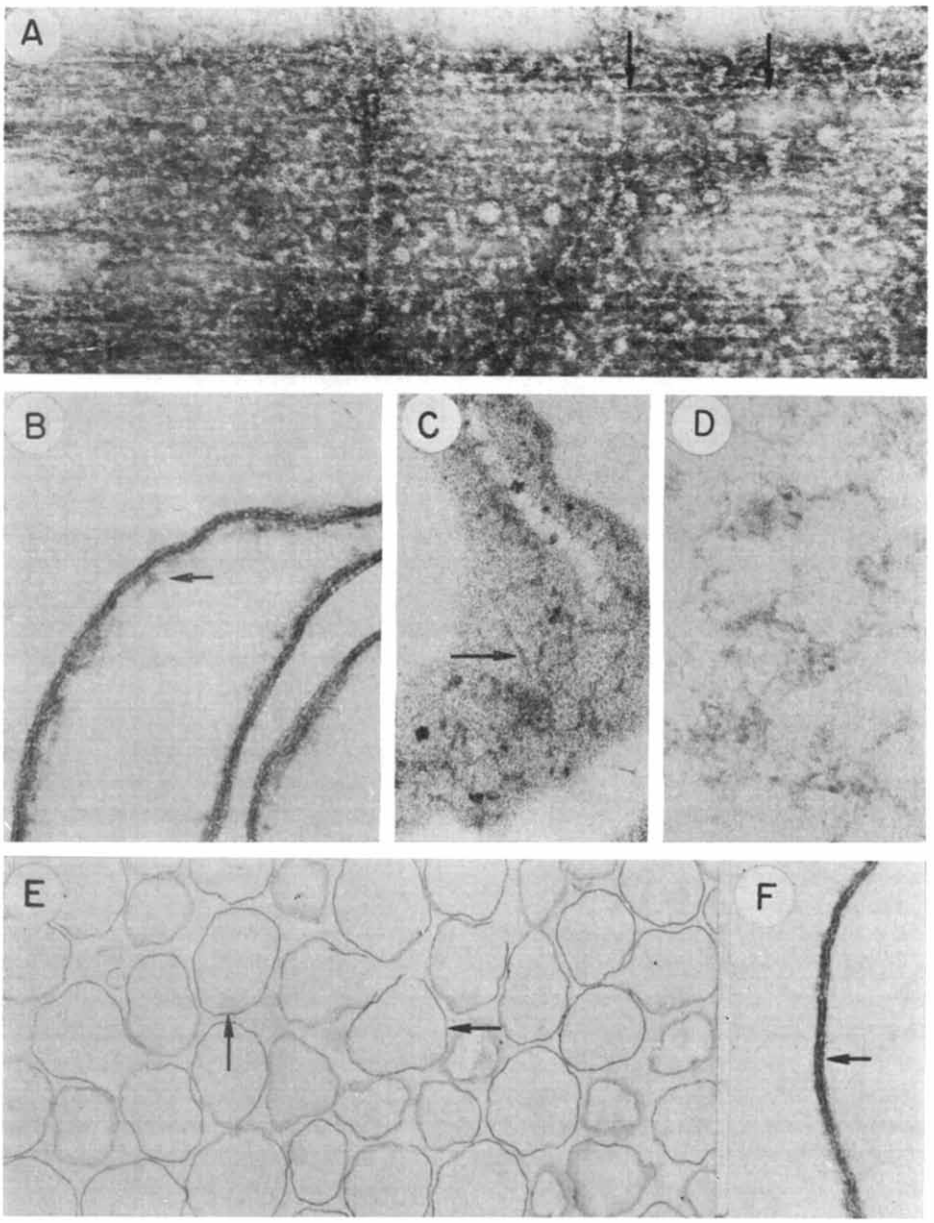

Fig. 4. Negative-stain preparation of specimens diluted in 0.5 mM Na_2EDTA (pH 7.4) and stained with 1 % uranyl formate. A. Soluble protein extract (0.1 mg/ml) in the presence of 2 mM $CaCl_2$. Note the fibrils with a diameter of about 40 Å (arrows). Magnifications × 135000. Thin sections of glutaraldehyde–osmium-fixed, epoxy-embedded specimens. Thin sections were stained with uranyl acetate and lead citrate. B. Intact erythrocyte ghosts. Note the fibrillar material present on the inner surface of the trilaminar unit membrane (arrow). Magnification × 135000. C. Tangential section of erythrocyte ghost membrane showing meshwork of fibrils in the plane of the membrane (arrow). × 135000. D. Protein extracted from erythrocyte ghosts by low-ionic-strength dialysis in 0.5 mM Na_2EDTA (pH 7.4) was gelled by the addition of 2 mM $CaCl_2$. Magnification × 135000. E. Ghost fragments formed during protein solubilization show closed (vertical arrow) and open (horizontal arrow) structures. Magnification × 43000. F. A higher magnification of a ghost fragment showing loss of fibrillar material from the inner surface of the unit membrane (arrow). Magnification × 135000.

the protein-rich supernatant while vesicular fragments of ghosts formed during protein solubilization did not have detectable fibrillar structures[26].

TABLE II

SUBSTRATE AND ASSAY SPECIFICITY FOR DETECTION OF Ca^{2+}-DEPENDENT ATPASE ACTIVITY

The reaction volume for the isotopic and colorimetric determinations were 100 μl and 1.5 ml, respectively. Each assay contained 6 mM $CaCl_2$, 1 mM Na_2ATP, 0.25 mM EDTA, and 25 mM HEPES buffer (pH 7.5). Incubation was at 37° for 60 min. Phosphate release was expressed as nmoles $P_i \cdot$mg protein$^{-1} \cdot h^{-1}$.

Substrate	*Ca^{2+}-dependent ATPase activity*	
	Colorimetric	*Isotopic*
ATP	17.4	15.4
GTP	nil	–

In an effort to determine the orientation of these fibrils on the unit membrane, thin sections of plastic-embedded ghosts were examined. In addition to the classic unit membrane structure, an irregular layer of finely fibrillar material was located adherent to the inner surface of plasma membrane (Fig. 4B). Sections of membrane which had been cut "face on" were then surveyed. A matrix of fibrils parallel to the unit membrane was identified (Fig. 4C) in those sections which presented a tangential view of the inner surface of the membrane. These fibrillar structures appeared to be contiguous with the inner membrane coat seen in cross-sectioned ghosts. Thin sections of a centrifuged pellet of cation-gelled protein were examined and fibrillar material morphologically similar to the cross-sectioned view of those structures lost from the inner surface of the ghost membrane during dialysis could be identified (Fig. 4D).

Thin sections of plastic-embedded ghost membrane fragments produced by dialysis at low ionic strength were also examined. These fragments appear in two forms, open membrane fragments and closed structures, presumable cross sections of spherical vesicles about 1.0 μ in diameter (Fig. 4E). These membranes have lost the fibrillar material associated with the inner surface on the intact ghost unit membranes, while retaining their trilaminar structure (Fig. 4F).

DISCUSSION

Ca^{2+}-dependent ATPase activity and membrane fibrils

Ca^{2+}-dependent ATPase activity has been described previously in human erythrocyte membranes[15–17]. The activity reported in these intact membrane systems has varied with the concentration of Ca^{2+}, Mg^{2+} and the manner in which the ghosts were prepared. WINS AND SCHOFFENIELS[18,19] have studied the divalent cation activity of human ghosts in detail. They have interpreted this dual requirement for Ca^{2+} and Mg^{2+} as indicating an interaction of these divalent cations with multiple sites on a single enzyme. Their data, however, do not exclude the presence of multiple Ca^{2+}-sensitive enzyme activities. Our data suggest that at least one component of the total Ca^{2+}-sensitive ATPase activity is Mg^{2+}-inhibited, albeit, a quantitatively minor contributor to the total activity.

Ca^{2+}-dependent ATPase activity in erythrocyte ghosts and in the vesicles formed upon protein solubilization was measured in the presence of ouabain (0.2 mM) by noting the difference in activity with and without added Ca^{2+}. It should be noted that other inhibitors of Ca^{2+}-sensitive enzymes, such as salygran were not tested. Variability of the non Ca^{2+}-dependent, ouabain-insensitive ATPase activity has prevented a precise determination of the Ca^{2+}-dependent component in ghosts and vesicles, as well as the percentage of this activity removed from the ghost membrane and associated with the solubilized protein and *in vitro* fiber system. WHEELER AND WHITTAM[20] reported similar difficulties in the measurement of Ca^{2+}-stimulated, ouabain-insensitive ATPase activity in the microsomal fragments of rabbit kidney cortex. Although the cause of this variability was not ascertained, differences in the permeability of the final membrane preparations to chelating agent, Ca^{2+} or Mg^{2+} may be involved.

In contrast, measurement of Ca^{2+}-dependent and independent ATPase activity in the protein-rich supernatant was reproducible from preparation to preparation and was not altered by ouabain. In the absence of Mg^{2+}, a minimum of 12 % of the total Ca^{2+}-activated ATPase activity of unaltered ghosts or 36 % of the total activity remaining after dialysis (vesicles *plus* soluble fraction) was associated with the solubilized protein fraction.

The measurement of Ca^{2+}-dependent ATPase activity by the method of BERENBLUM AND CHAIN[14] excludes the possibility that the increase in ^{32}P counts detected with the radioassay was due to a Ca^{2+}-mediated phosphate exchange reaction. The colorimetric method of analysis, however, required excessive quantities of protein and was not used routinely.

It is unlikely that the Ca^{2+}-dependent ATPase activity measured represents contaminating membrane fragments since a partially purified preparation of the supernatant which contains essentially all of the isolated fibrils and ATPase activity contains less than 1 % lipid and 0.1 % carbohydrate[26]. In addition, no membrane fragments were found when negatively stained preparations of this fraction were examined in the electron microscope.

The solubilized protein consists of several components[26]; the possibility remains therefore that the Ca^{2+}-dependent ATPase activity measured is attributable to only a fraction of the total protein isolated. Although the isolated fiber system as seen with negative staining and those fibrils seen on the intact ghost membrane are morphologically similar, such similarities should not be interpreted as evidence for chemical identity. The observation that the isolated fiber system and Ca^{2+}-dependent ATPase activity separate together on a Sepharose 4B column suggests that there is an association between these two parameters. To establish that the protein or proteins which form fibrils are identical to those responsible for ATPase activity will require the independent demonstration in both systems (intact membranes and protein extract *in vitro*) that fibril formation and ATP hydrolysis are functions of a single protein or protein complex.

It should be noted that inner membrane fibrils have been described previously in electron micrographs of thin-sectioned membranes of human[21] and guinea pig[22] erythrocyte ghosts. Fibrillar structures extending 400–500 Å into the cytoplasm of freeze-etched nucleated erythrocytes were also found by KOEHLER[23]. Two observations in this study suggest that the fibrils seen in cross-sectional views of the mem-

brane are identical to the reticular fibrils described when the membrane is appropriately negatively stained. First, the reticular fibrils seen in tangentially sectioned membrane are contiguous with the inner membrane fibrils and appear morphologically similar to thin-sectioned cation-gelled protein. Second, cation-gelled protein when negatively stained appears as fibrils structurally similar to those found on native ghost membranes and solubilized by low-ionic-strength dialysis. Thus, these fiber systems may represent different views of a single membrane structure located *in situ* on the inner aspect of the erythrocyte membrane and obtained upon isolation and reexposure to salt in the form of fibrils.

Ca^{2+}, ATP and erythrocyte deformability

It seems reasonable to speculate on what physiologic role a system of membrane fibrils may play in erythrocyte function. The erythrocyte membrane is deformable and as such exhibits elasticity. This property of elasticity is intrinsic to the plasma membrane[24]. LACELLE AND WEED[25] have recently presented data which indicates that the inclusion of 1 mM ATP and Mg^{2+} during hypotonic hemolysis of human erythrocytes is required for the maintenance of ghost deformability while as little as 0.1 mM Ca^{2+} rendered the human ghost rigid. A Ca^{2+}-dependent, Mg^{2+}-inhibited ATPase associated with a network of membrane fibrils could represent a mechanochemical basis for such deformability.

Evidence that some component of the solubilized protein may contribute to this property of membrane elasticity and that the intact ghost is capable of "contraction" will be presented elsewhere[26]. Additional support for the concept that membrane fibrils contribute to membrane elasticity is found in the morphologic observations of the vesicular fragments that remain subsequent to low-ionic-strength dialysis and protein solubilization. When examined in the electron microscope, thin sections of these vesicular structures (Fig. 4E) which have lost their membrane fibrils and Ca^{2+}-dependent ATPase activity on protein solubilization, exhibited open-ended, broken membranes. Free-ended membranes are rarely observed in samples of ghosts or other membrane preparations. It is not unreasonable to suggest that an alteration of the physical properties of these membranes (*i.e.* the loss of an elastic component) has occurred. Vesicles formed by sonication of ghosts on the other hand retain their inner membrane fibrils and do not exhibit broken edges[22].

Further studies of the relationship of this Ca^{2+}-dependent, Mg^{2+}-inhibited ATPase activity to the reticular fibrils on ghost membranes may provide insight into the mechansims by which membrane elasticity and rigidity are maintained.

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