STUDIES ON THE REACTION CYCLE OF THE CALCIUM TRANSPORT ATPASE FROM HUMAN ERYTHROCYTES

by

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## ABSTRACT

The plasma membrane calcium-transport ATPase plays a major role in maintaining the low cytosolic calcium concentrations required for normal cellular function. Calcium, magnesium, calmodulin and lanthanum have been shown to alter the activity of the calcium-stimulated, magnesium-dependent ATPase activity in human erythrocytes. In an attempt to examine the reaction sequence of the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase, the effects of these agents on the kinetics of calcium dependent phosphoprotein formation, the first step in the partial reaction sequence, were examined. Calmo-dulin-depleted erythrocyte membranes were prepared by hypotonic lysis in the presence of EDTA, according to the method of Carafoli et al (1980).

Calcium-dependent formation of the phosphorylated intermediate was biphasic; the high calcium-affinity component was associated with low levels of E.Ca.P and a shallow response to changing calcium concentrations, whereas in the region of the low calcium-affinity component, E.Ca.P rose sharply in response to increasing calcium concentrations. The low affinity component of E.Ca.P lies in the range of calcium concentrations which inhibit $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase activity. When analyzed on LiDS acid PAGE, both components of calcium-dependent phosphoprotein formation were due to hydroxylamine-sensitive phosphorylation of a 135,000145,000 dalton protein. Hence, the low calcium-affinity component of phosphoprotein formation and calcium-dependent inhibition of $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase activity were likely due to calcium-
inhibition of dephosphorylation. Kinetic studies of calciumdependent phosphoprotein formation, at two different calcium concentrations ( $1.0 \mu \mathrm{M}, 0.4 \mathrm{mM}$ ), indicated that a steady-state was reached much sooner at higher calcium concentrations. Lanthanum, which is known to block dephosphorylation of the intermediate complex, increased both the apparent rate of formation and the steady-state level of the phosphorylated intermediate. Calmodulin, which has previously been shown to increase both the maximum velocity and the calcium affinity of the $\left(\mathrm{Ca}^{2+}+\right.$ $\mathrm{Mg}^{2+}$-ATPase, did not affect either calcium-dependent inhibition of $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase activity or the biphasic nature of cal-cium-dependent phosphoprotein formation. At low calcium concentrations, calmodulin increased the apparent rate of phosphoprotein formation, whereas at higher calcium concentrations ( 0.4 mM ) calmodulin reduced the steady-state level of the phosphoprotein; the apparent rate of formation was unaffected. In the presence of lanthanum, calmodulin increased both the apparent rate of formation and steady-state level of the phosphoprotein, suggesting that the true rate of formation was increased by calmodulin at higher calcium concentrations, but this was normally hidden by a simultaneous increase in the rate of dephosphorylation.

Removal of endogenous magnesium, using trans-1,2-diaminocyclohexane tetraacetic acid (CDTA) did not alter the calcium sensitivity or rate of formation of the phosphorylated intermediate, however turnover of the intermediate was markedly reduced. In the absence of free magnesium, both the velocity and calcium
sensitivity of the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase were also found to be lower.

The low calcium-affinity component of calcium-dependent phosphoprotein formation, which Schatzmann (1982) has attributed to an action of calcium at a "magnesium-specific" site, was not affected by magnesium concentrations as high as 1 mm . Furthermore, this phosphoprotein could be dephosphorylated along either the forward or reverse pathways. These results indicate that the transformation from $E_{1}$. Ca.P to $E_{2}$.Ca.P may not be the site of the calcium-dependent inhibition of dephosphorylation.

Calmodulin-depleted membrane fragments were prepared from the erythrocytes of cystic fibrosis patients as well as age- and sex-matched controls. Under conditions in which dephosphorylation is inhibited, phosphoprotein formation and $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase activities were determined. Both $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase activity and phoshoprotein formation were found to be significantly reduced in the preparations derived from patients with cystic fibrosis. Turnover of the phosphorylated intermediate did not differ significantly between the two groups. A reduction in $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase activity and phosphoprotein formation suggests that there may be fewer active calcium pumping sites in the erythrocyte membranes of cystic fibrosis patients compared to normal subjects.
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| ADP | adenosine 5'-diphosphate |
| :---: | :---: |
| AMP | adenosine $5^{\prime \prime}$-monophosphate |
| ATP | adenosine $5^{\prime \prime}$-triphosphate |
| ${ }^{\circ} \mathrm{C}$ | degrees Centigrade |
| $\mathrm{Ca}^{2+}$ | free (ionized) calcium |
| $\begin{array}{r} \mathrm{Ca}^{2+}+ \\ \text { ATPas } \end{array}$ | - calcium-stimulated, magnesium-dependent ATPase |
| Ci | Curie |
| CAMP | adenosine $3^{\prime}: 5^{\prime}$-cyclic monophosphate |
| CDTA | trans-1,2-diaminocyclohexane-N,N, $\mathrm{N}^{\prime}, \mathrm{N}^{\prime},-$ tetraacetic acid |
| cpm | counts per minute |
| CTP | cytidine 5'-triphosphate |
| dpm | decays per minute |
| $\mathrm{E}_{1} . \mathrm{Ca} \cdot \mathrm{P}$ | phosphorylated enzyme-calcium complex in the $\mathrm{E}_{1}$ state |
| $\mathrm{E}_{2} \cdot \mathrm{Ca} \cdot \mathrm{P}$ | phosphorylated enzyme-calcium complex in the $\mathrm{E}_{2}$ state |
| EDTA | ethylenediaminetetraacetic acid |
| EGTA | ethyleneglycolbis-( beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid |
| et al | and others |
| f | femto |
| 9 | gram |
| GTP | guanosine 5'-triphosphate |
| $3^{3}$ | tritium |
| HEPES | $\begin{aligned} & \text { (N-2-hydroxyethylpiperazine-N'-ethanesulfonic } \\ & \text { acid } \end{aligned}$ |
| ITP | inosine 5'-triphosphate |


| $\mathrm{K}_{\mathrm{d}}$ | dissociation constant |
| :---: | :---: |
| 1 | liter |
| Lids | lithium dodecylsulphate |
| m | milli |
| M | molar |
| mA | milliamperes |
| min | minute |
| mg | milligrams |
| mol | mole |
| $\mu$ | micro |
| $\begin{aligned} & \mathrm{Na}^{+}, \mathrm{K}^{+}- \\ & \text {ATPase } \end{aligned}$ | sodium-, potassium-stimulated ATPase |
| 32 P | phosphorus-32 |
| PAGE | polyacrylamide gel electrophoresis |
| pmol | picomoles |
| $\mathrm{P}_{\mathrm{i}}$ | inorganic phosphate |
| SDS | sodium dodecylsulphate |
| S.E.M. | standard error of the mean |
| S.R. | sarcoplasmic reticulum |
| TCA | trichloroacetic acid |
| TEMED | N, N, $N^{\prime}$, $N^{\prime}$-tetramethylethylenediamine |
| Tris | tris (hydroxymethyl) aminomethane |
| UTP | uridine 5'-triphosphate |
| $\mathrm{V}_{\text {max }}$ | maximum velocity |
| $\mathrm{V}_{\text {obs }}$ | observed velocity |
| w/v | weight per unit volume |
| 1 | per |
| \% | percent |

DEDICATION

To my parents.

I am deeply grateful to my supervisors, Dr. S. Katz and Dr. B.D. Roufogalis, for their guidance and support throughout this study.

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Finally, I wish to thank all members of the faculty, staff and graduate student body in the Faculty of Pharmaceutical Sciences, U.B.C. for making this Masters program enjoyable.
I. Role of Calcium in Modulating Intracellular Activities.

Recently calcium has been recognized as a key factor in regulating many cellular functions. The action potential in a nerve cell is triggered and modulated by an influx of calcium across the axonal membrane (Baker, 1972). Muscle contraction is evoked by calcium release from membrane-associated calcium pools, such as the sarcoplasmic reticulum, into the cytosol (Ebashi, (1976). In mediating cellular responses to electrical or a wide variety of chemical stimuli, calcium may function either; 1) alone as an intracellular "second messenger", or 2) by affecting enzyme activities such as adenylate cyclase and/or phosphodiesterase to modulate the second messenger role of cyclic AMP (Rasmussen and Goodman, 1977). As a second messenger calcium elicits a physiological response via calcium-modulated proteins. These proteins are not enzymes themselves but serve to transmit the calcium "message" through calcium-dependent interactions with target enzymes and structural proteins. One such protein is calmodulin, which undegoes a conformational change upon binding calcium, allowing it to interact with, and thereby regulate the activity of a wide variety of target enzymes (for review see Scharff, 1981). Stimulus-evoked redistribution of intracellular calcium is known to regulate various contractile and secretory systems, membrane and other cellular events in addition to activating or inhibiting a wide variety of specific enzyme systems (Case, 1980).

Due to the specific nature of the interactions involved in calcium fluxes and binding to target proteins, the biologically active form of calcium is its ionized or "free" state. All of the regulatory functions of calcium are based upon the existence of a steep electrochemical gradient for calcium which is oriented towards the cytoplasm. The concentration of free calcium ions in the cytoplasm is typically less than $10^{-5}-10^{-7} \mathrm{M}$, whereas much higher calcium levels (about $10^{-3} \mathrm{M}$ ) are found in extracellular fluids and in intracellular organelles such as the mitochondria and the sarcoplasmic reticulum (SR).

The protein targets for calcium are located either in the cytoplasm or on the membranes exposed to the cytoplasm. Onset of the physiological response is triggered by an increase in the cytoplasmic concentration of free calcium ions. The duration of the response is often dependent on the duration of the calcium "pulse"; for example the contraction of striated muscle terminates as the concentration of calcium in the cytoplasm decreases. In addition, for calcium to act as a second messenger in more than one response within the same cell, changes in cytosolic calcium concentration have to be compartmentalized. The stimulus-induced rise in cytosolic calcium must not result in a uniform increase in cytosolic calcium or there would be no selectivity in the response elicited. Hence a means whereby calcium levels are rapidly restored to their low resting levels following a stimulus is essential in order to terminate calciumdependent events and to allow calcium to function as a selective second messenger.
II. Regulation of Intracellular Free Calcium Concentrations

A large electrochemical gradient for calcium is thermodynamically unfavorable and therefore must be maintained at the expense of energy by the cell. Low cytoplasmic calcium concentrations are achieved by binding of calcium to sites within the cell and by energy-dependent calcium transporting systems in both the plasma membrane and intracellular organelles. Intracellular calcium binding sites include negatively charged phospholipids associated with the various membrane systems and a group of macromolecules referred to as calcium binding proteins. There are two types of active transport mechanisms associated with calcium metabolism: 1) $\mathrm{Na}^{+}-\mathrm{Ca}^{2+}$ exchange, (Schatzmann, 1975; Sarkadi and Tosteson, 1979), where calcium is extruded in exchange for inwardly moving sodium ions thereby exploiting energy originally invested in the sodium gradient by the $\mathrm{Na}^{+}, \mathrm{K}^{+}$-ATPase; and, 2) Calcium pumping, catalyzed by a specific family of enzymes, at the expense of metabolic energy in the form of ATP. Sodiumcalcium exchange occurs in plasma membranes (with the exception of the erythrocyte) whereas, in addition, calcium pumping enzymes are found in the plasma membrane, sarcoplasmic reticulum, and mitochondrial membranes. These are independent calcium transport systems, molecularly and possibly mechanistically different from one another.

Intracellular organelles are well suited for rapid regulation of cytosolic calcium levels since their total calciumtransporting membrane area is generally much greater than that of
the plasma membrane. Although estimates of free calcium concentrations in these organelles range from $10^{-6}-10^{-5} \mathrm{M}$, their total calcium content is in the millimolar range due to extensive calcium buffering capabilities. In the mitochondria, calcium combines with inorganic phosphate to form an osmotically inactive calcium phosphate complex. In the sarcoplasmic reticulum, calcium appears to bind to calsequestrin, a low-affinity ( $K_{d}=0.8 \mathrm{mM}$ ) high capacity calcium binding protein (Ostwald and MacLennan, 1974). This process increases the capacity of the organelle for calcium uptake. Calcium transport into the mitochondria represents a special case with respect to ATP-driven calcium fluxes. The actual driving force for calcium movement appears to be the membrane potential created by the extrusion of protons by an ATPase (Carafoli, 1974; Bygrave, 1978; Carafoli and Crompton, 1978). The calcium "pump" of mitochondrion has a $K_{d}$ for calcium of roughly $10^{-5} \mathrm{M}$ (Crompton et al, 1976; Carafoli, 1982) whereas for the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase in S.R. in skeletal muscle the $K_{d}$ has been estimated to be $3-5 \times 10^{-7} \mathrm{M}$ (deMeis, 1971; Kanazawa et al, 1971; Meisner, 1973; Ikemoto, 1974) and $1-2 \times 10^{-6} \mathrm{M}$ in cardiac muscle (Shikegawa et al, 1976). In view of its low affinity for calcium there is some question as to the extent to which calcium uptake into the mitochondria is involved in maintaining low cytosolic calcium concentrations in the unstimulated cell. There have been suggestions that the function of the mitochondrial calcium transport system is to regulate the calcium concentration within the mitochondria itself, not that of the cytoplasm (Denton and McCormack, 1980). However, Penniston
(1983) has suggested that the mitochondria may primarily be a back-up system designed to limit large increases in cytoplasmic free calcium levels. Though mechanisms such as calcium binding and calcium storage organelles are suitable for rapid removal of small amounts of calcium from the cytoplasm they would not be able to maintain a low calcium concentration in the cytoplasm indefinitely. The extracellular calcium which enters the cell in response to each stimulus or action potential would rapidly saturate these sites. Penniston (1983) has suggested that the role of the plasma membrane calcium transport mechanisms is to recycle that portion of the cytoplasmic calcium that is extracellular in origin, so as to prevent a build-up of calcium within the cell.

There is still some controversy regarding the relative importance of the calcium pump with respect to $\mathrm{Na}^{+}, \mathrm{Ca}^{2+}$-exchange in maintaining a low cytoplasmic free calcium concentration. Studies conducted in both the squid axon (Dipolo and Beauge, 1979) and sarcolemmal vessicles isolated from dog heart (Caroni and Carafoli, 1981) have suggested that ATP-dependent calcium efflux is dominant at the physiological resting calcium concentration, whereas the $\mathrm{Na}^{+}, \mathrm{Ca}^{2+}$-exchange is effective only at much higher concentrations, such as might occur during stimulation.

## III The Plasma Membrane Calcium Pump

The plasma membrane calcium pump and its associated $\left(\mathrm{Ca}^{2+}+\right.$ $\mathrm{Mg}^{2+}$ )-ATPase activity have been particularly well characterized in the human erythrocyte, as these cells provide a unique system
for studying the regulation of this enzyme. Erythrocytes do not contain calcium-accumulating organelles or a $\mathrm{Na}^{+}, \mathrm{Ca}^{2+}$-exchange mechanism, yet they maintain a low intracellular free calcium concentration and they can be obtained in a relatively pure and homogeneous form using mild isolation procedures. For these reasons erythrocytes provide a readily available source of plasma membrane $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase which is free from contamination by other species of calcium ATPases.

Dunham and Glynn (1961) were the first to demonstrate the presence of a ouabain-insensitive magnesium-dependent, calciumstimulated adenosine triphosphatase activity in the plasma membrane of human erythrocytes. This ATP-hydrolytic activity was several-fold more active than the ouabain-sensitive $\mathrm{Na}^{+}, \mathrm{K}^{+}$-ATPase activity in these membranes. A calcium transport activity was reported in the erythrocyte plasma membrane five years later by Schatzmann (1966) and it was shown that this calcium pumping mechanism was closely coupled to the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase activity (Schatzmann and Vincenzi, 1969; Lee and Shin,1969). It is now well established that the splitting of ATP to ADP and $P_{i}$ is necessary to produce active calcium transport (Schatzmann, 1975; Sarkadi and Tosteson, 1979).

The plasma membrane calcium pump exists as an intrinsic membrane protein with a molecular weight of 135,000-145,000 daltons (Knauf et al, 1974; Katz and Blostein, 1975; Schatzmann and Burgin, 1978; Lichtner and wolf, 1979; Carafoli et al, 1982) which, in light of its function, probably spans the plasma membrane. Studies of the purified enzyme using polyacrylamide gel
electrophoresis also show evidence of a higher molecular weight peptide (Niggli et al, 1979; Schatzmann, 1982) which is thought to represent a dimer of the enzyme (Hinds et al, 1982). In addition, results from radiation inactiviation studies indicated that the enzyme functions as a dimer in the plasma membrane (Minocherhomjee et al, 1983). The significance of these observations, regarding the true functional unit of the calcium pump, is still unknown. The molecular weight of the calcium pump protein from sarcoplasmic reticulum has been estimated to be between 100,000 and 115,000 daltons (Inesi et al, 1970; MacLennan, 1970; Louis and Shooter, 1972; Thorley-Lawson and Green, 1975; LeMaire et al. 1976; Rizzolo et al, 1976), emphasizing the fact that the calcium transport mechanisms found in these two membrane systems are different.

The measured activity and calcium sensitivity of the $\left(\mathrm{Ca}^{2+}+\right.$ $\mathrm{Mg}^{2+}$ )-ATPase varies depending upon the method of membrane preparation, the nature of the substrate and cofactors added to the assay medium and the presence of regulators (Roufogalis, 1979). The substrate of the calcium pump is ATP and its cofactors include magnesium, calcium and possibly potassium. The best known regulator of the erythrocyte calcium pump is calmodulin, however $A u$ (1978) has recently discovered a protein which inhibits the activity of the calcium pump.

## 1. Substrates

Active transport of calcium requires ATP as a substrate. Other nucleoside triphosphates (CTP, GTP, ITP, UTP) may substi-
tute for ATP (Lee and Shin, 1969; Olson and Cazort, 1969), however the enzyme demonstrates a higher specificity for ATP (Cha et al, 1971; Sarkadi et al, 1979). Other phosphate esters, such as ADP, AMP, pyrophosphate, acetyl phosphate, or nitrophenyl phosphate cannot support active calcium transport (Olson and Cazort, 1969; Schatzmann and Vincenzi, 1969; Sarkadi et al, 1979; Caride et al, 1983).

There is some controversy as to the actual species of ATP which provides the energy for calcium transport. The proposed substrates include: Mg-ATP (Wolf, 1972; Wolf et al, 1977); Ca-ATP (Penniston et al, 1980; Graf and Penniston, 1981a); and free ATP (Rega and Garrahan, 1975; Schatzmann, 1977; Richards et al, 1978; Sarkadi et al, 1978). More recent studies tend to suggest that more than one of these forms of ATP may act as the substrate. Muallem and Karlish (1981) have suggested that Mg-ATP, Ca-ATP and free ATP are equally effective whereas Penniston (1982b) proposed that Mg-ATP is the substrate but that Ca-ATP can also be utilized in the absence of magnesium. In short, there is not a good consensus on the true physiological substrate(s) for calcium transport.

ATP activation of both calcium transport (Muallem and Karlish, 1979; Mollmán and Pleasure, 1980) and its associated $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase activity (Richards et al, 1978; Muallem and Karlish, 1979, 1980; Steige and Luterbacher, 1981) follows a biphasic curve, showing both a "high-" and a "low-affinity" component for $\operatorname{ATP}\left(K_{\text {ATP, } 1}=1-2 \times 10^{-6} \mathrm{M}, \mathrm{K}_{\mathrm{ATP}, 2}=2-3 \times 10^{-4} \mathrm{M}\right)$. These results have been taken to mean that there are two binding
sites for ATP of different affinities. Muallem and Karlish (1978) suggested that at the high-affinity binding site ATP acts as the substrate for transport, while higher ATP concentrations may regulate the activity of the enzyme through an allosteric effect at the low-affinity binding site. As an alternate interpretation, Neet and Green (1977) showed that these results could equally well be explained in terms of a single site having different affinities in two states of the reaction cycle.

In both intact erythrocytes and resealed ghosts active calcium extrusion requires intracellular ATP, whereas calcium uptake into inside-out vesicles requires the presence of ATP in the incubation medium (Sarkadi, 1980). These results show that ATP interacts at a site on a region of the enzyme, normally facing the cytoplasm, which does not cross the membrane during the translocation of calcium. The inorganic phosphate ( $\mathrm{P}_{\mathrm{i}}$ ) produced from the enzymatic hydrolysis of ATP to ADP is not co-transported with calcium, but released back into the cytoplasm (Olson and Cazort, 1974; Schatzmann and Roelofsen, 1977).

## 2. Effectors

a. Calcium. As the role of the calcium pump is to move calcium from a region of low calcium concentration (the cytoplasm) to a region of considerably higher calcium concentration (the extracellular medium) the pump should have a high affinity for calcium on the intracellular side of the membrane and a much lower affinity on the outer face of the membrane. At the internal membrane surface, calcium activates the calcium transport
system and its associated $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase activity while showing almost no effect at the external surface (Sarkadi, 1980). To overcome difficulties in correctly estimating the free calcium concentraion, EGTA is added as a calcium buffer. The free calcium concentration is calculated from the dissociation constants of all possible chelators of calcium present in the assay medium after correction for assay conditions such as temperature and ionic strengh. When measured in the presence of a Ca-EGTA buffering system the affinity for calcium at the internal surface is roughly $1-5 \times 10^{-6} \mathrm{M}$ (Schatzmann, 1973; Schatzmann, 1982; Penniston, 1983). There have been some doubts regarding the validity of dissociation constants for calcium which are esimated in the presence of EGTA. This anion has been reported to increase the calcium sensitivity of calcium uptake into inside-out vesicles (Sarkadi et al, 1973) and on the ( $\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}$ )-ATPase activity in resealed ghosts (Schatzmann, 1973), in intact erythrocyte membranes (Orlov and Schevchenko, 1978; Al-Jobore and Roufogalis, 1981a) and in Triton $X-100$ solubilized preparations (Al-Jobore and Roufogalis, 1981a; Al-Jobore et al, 1981). Recently, however, Muallem and Karlish (1982) demonstrated that the affinity of the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase in intact erythrocytes is high $\left(K_{d, C a}=2 \times 10^{-7} \mathrm{M}\right)$. This finding corroborates previous determinations of calcium affinity which employed EGTA, but leaves open questions concerning the nature of the low calcium sensitivity expressed in other preparations in the absence of EGTA.

In addition to its stimulatory action upon the $\left(\mathrm{Ca}^{2+}+\right.$
$\mathrm{Mg}^{2+}$ )-ATPase calcium, in excess amounts (above $1 \times 10^{-4}-5 \times 10^{4}$ M), will inhibit the activity of this enzyme (Schatzmann, 1982); this effect is thought to occur at an inhibitory site on the enzyme (Klinger et al, 1980; Vincenzi et al, 1980; Graf and Penniston, 1981). Partial digestion of the enzyme with trypsin, which cleaves a regulatory domain off of the enzyme, does not affect calcium-dependent inhibition (Steiger and Schatzmann, 1981; Roufogalis et al, 1982). These results indicate that the site of inhibition is likely to be in the catalytic domain. A possible explanation for this effect of calcium is discussed below.

There is some controversy in the literature concerning the true stoichiometry of the plasma membrane calcium pump. Hill coefficients for calcium range from around 1 (Schatzmann, 1973; Schatzmann and Roelofsen, 1977) to around 2 (Quist and Roufogalis, 1977; Scharff, 1978; Akyempon and Roufogalis, 1982). Ferrierra and Lew (1976) have shown that 2 calcium ions participate in the reaction cycle. Direct measurement of the stoichiometry reconstituted vesicles (Niggli et al, 1982) suggest a Ca/ATP ratio of $1 / 1$. Schatzmann (1985) has pointed out that a stoichiometry of 2 is not thermodynamically feasable whereas a one to one ratio would be acceptable. However, this assumes an electrogenic mode of operation of the pump, whereas the countertransport of one or two protons per calcium ion would alter this restriction (Tanford, 1984).
b. Magnesium. Magnesium has several roles in regulating $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase activity. Although not transported by the
calcium pump, magnesium must be present in intact cells and resealed ghosts to observe optimum active calcium extrusion (Schatzmann and Vincenzi, 1969; Lee and Shin, 1969; Olson and Cazort, 1969). In inside-out vesicles active calcium uptake requires magnesium on the cytosolic side of the vesicles (Weiner and Lee, 1972; MacIntyre and Green, 1977; Sarkadi et al, 1978; Cha et al, 1971). These results suggest that of the ATP- and $\mathrm{Mg}^{2+}$-binding sites are located on the same side of the membrane, namely on the cytosolic surface of the membrane. Although this enzyme was originally thought to be absolutly dependent upon the presence of magnesium for activity (Dunham and Glynn, 1961; Lee and Shin, 1969; Shatzmann, 1975) more recent reports indicate that this is not the case. Richards et al (1978) found that a small calcium-stimulated ATPase activity remained in the absence of magnesium and that while magnesium enhanced the stimulatory effect of calcium, it had no effect on the affinity for calcium or ATP. The stimulatory action of magnesium on the ATPase followed simple saturation kinetics $\left(K_{d, M g}=0.33 \mathrm{~mm}\right)$ (Richards et al, 1978).

Magnesium concentrations in the millimolar range compete with calcium, shifting the calcium concentration which produces $1 / 2$ maximum activation of the ATPase to higher values in erythrocyte ghosts (Klinger et al,1980; Al-Jobore and Roufogalis, 1981b; Scharff, 1980), Triton $X-100$ solubilized membranes (Al-Jobore and Roufogalis, 1981b), in inside-out vesicles (Akyempon and Roufogalis, 1982) and in the purified enzyme (Villalobo et al, 1985). Klinger et al (1980) found that magnesium also antag-
onized calcium-dependent inhibition of the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase. Al-Jobore and Roufogalis (1981b) suggested that magnesium allows the binding of activating calcium but prevents the binding of calcium at an inhibitory site on the enzyme. Schatzmann (1982) proposed a similar idea, suggesting that at high concentrations, calcium became inhibitory by way of a competition at a magnesiumspecific site.
c. Monovalent Cations. Although the calcium pump is not dependent on the presence of monovalent cations for activity (Schatzmann, 1975), both calcium transport and its associated $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase activity are 30-50\% higher when measured in sodium or potassium containing solutions than in choline, tris or sucrose media (Schatzann and Rossi, 1971; Sarkadi et al, 1978). A similar effect has also been shown in purified enzyme preparations (Graf et al, 1982). $\mathrm{Rb}^{+}$or $\mathrm{NH}_{4}{ }^{+}$will mimic the stimulatory effects of $\mathrm{Na}^{+}$and $\mathrm{K}^{+}$but $\mathrm{Li}^{+}$will not (Wolf et al, 1977). The affinities for sodium and potassium differ markedly for this effect $\left(K_{N a}=20-30 \mathrm{mM}, K_{K}=4-6 \mathrm{mM}\right)$ (Schatzmann and Rossi, 1971; Scharff, 1978; Kratje et al, 1983). Kratje and coworkers (1983) recently showed that monovalent cations appear to exert their effects on the internal side of the membrane. This observation makes it unlikely that this stimulation of calcium transport is due to a counter-transport mechanism. Although calmodulin has been shown to enhance the stimulatory effect of potassium on the rate of ATP hydrolysis by the ( $\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}$ )-ATPase, (Scharff, 1978), this stimulatory effect of monovalent cations is independent of that produced by calmodulin (Sarkadi et al, 1980). It is
questionable that this represents a regulatory mechanism for the calcium pump, as the cytosolic concentrations of potassium and sodium exceed that required to obtain this effect.

## 3. Modulators

a. Calmodulin. Calmodulin, a heat-stable, acidic, calcium binding protein with a molecular weight of approximatly 16,800 daltons (Klee and Vanaman, 1982), has been isolated from a number of tissues, including brain, heart and testis. Initially characterized as an activator of phosphodiesterase, calmodulin has since been shown to be an intracellular modulator of a number of calcium-dependent enzymes. Each calmodulin molecule can bind 3-4 calcium ions with dissociation constants in the range of $10^{-6}$ -$10^{-4} \mathrm{M}$ (Wolff et al, 1972). The binding of calcium alters the conformation of calmodulin, increasing its helical content (Dedbman et al, 1977) and exposing hydrophobic regions (Laporte et al, 1980). In this conformation the calcium-calmodulin complex is able to bind to target enzymes (Niggli et al, 1979) and increase or decrease their activity.

The first observation that calmodulin was involved in regulating calcium fluxes across membranes was that of Bond and Clough (1973), who reported that a factor in hemolysate from human erythrocytes enhanced the activity of the erythrocyte $1 \mathrm{Ca}^{2+}$ $+\mathrm{Mg}^{2+}$ )-ATPase. Kinetic analysis of the calcium activation of the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase activity in isolated erythrocyte membranes revealed the presence of two $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase activi-
ties. Scharff (1972) had shown that erythrocyte "ghosts" prepared in the presence of calcium had higher $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase activity and affinity for calcium (Scharff, 1976) than those prepared in the presence of the calcium chelator, EDTA. These two activities were referred to as high and low affinity ( $\mathrm{Ca}^{2+}+$ $\mathrm{Mg}^{2+}$ )-ATPase, reflecting their different affinities for calcium (Horton et al, 1970; Schatzmann and Rossi, 1971; Scharff, 1972). Quist and Roufogalis (1975) showed that incubation of erythrocyte membranes in the presence of EDTA converted the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase from a high-affinity state to a low-affinity state; readdition of the extract restored high-affinity ATPase activity. These results suggested the action of a regulatory factor instead of two distict types of $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase. A cytosolic factor was identified as calmodulin (Gopinath and Vincenzi, 1977; Jarret and Penniston, 1977) and shown to regulate a reversible shift of the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase between low affinity/low activity and high-affinity/high activity states of the enzyme (Scharff and Foder, 1978). Calmodulin binding to the enzyme results in a 3-4 fold increase in the apparent maximum velocity and a 30 fold increase in the affinity for calcium at the transport site (Roufogalis and Mauldin, 1980; Foder and Scharff, 1981; Scharff and Foder, 1978, 1982).

In the presence of calcium, calmodulin binds very tightly to human erythrocyte membranes (Graff et al, 1980). The dissociation constant for calmodulin is $25 \mu \mathrm{M}$ in the presence of $0.1 \mu \mathrm{M}$ calcium (Scharff and Foder, 1982). Increasing the calcium concentration to $20 \mu \mathrm{M}$ reduces the dissociation constant (increases the affinity) of calmodulin to 2.5 nM (Scharff and Foder, 1982).

These authors found that the rate constant for the association of calmodulin with the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase is dependent on the calcium concentration. Hence, at low calcium concentrations a delay in the activation of the calcium pump may be expected. This delay has been observed experimentally (Vincenzi et al, 1980; Scharff and Foder, 1982). Scharff and coworkers (1983) suggested that such a mechanism provides a system whereby an abrupt rise of the calcium concentration within a cell will induce a slow association between calmodulin and the calcium pump, resulting in an activation with a lag which allows short pulses of calcium entry into a cell to produce a prolonged rise in cytosolic calcium. Without this delay, pulses of calcium entry would be terminated so rapidly that no effector mechanisms would be activated.

Brief exposure of human erythrocyte membranes to trypsin activates both the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase (Taverna and Hanahan, 1980) and calcium transport activity (Enyedi et al, 1980; Sarkadi et al, 1980). This effect resembles the effect of calmodulin in that both maximum velocity and calcium sensitivity are increased. Following tryptic digestion, these membranes are no longer activated by the addition of exogenous calmodulin. Sarkadi and coworkers (1980) proposed that calmodulin binds to a "subunit" of the enzyme, on the cytoplasmic surface of the membrane, and that this subunit of the enzyme functions as an inhibitor of the calcium pump. Tryptic cleavage or calmodulin binding relax the constraining effect of this inhibitory subunit, thereby increasing both the calcium affinity and the maximum
velocity of the calcium pump.
b. Endogenous Inhibitory Protein. Evidence now suggests that in addition to a stimulator of the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase there is also an inhibitor present in the cytosol of erythrocytes. Concentrated membrane-free erythrocyte cytoplasm has been shown to inhibit both $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase activity (Au, 1978) and calcium transport (Sarkadi et al, 1980) in erythrocyte membranes. Lee and $A u$ (1981) have recently isolated a protein from the hemolysate of pig erythrocytes which demonstrates these properties. This protein has an apparent molecular weight of 7,500 daltons and is partially inactivated by trypsin, lyophylization and heat. Calcium concentrations up to $5.0 \times 10^{-4} \mathrm{M}$ do not interfere with the inhibitory actions of this protein. The physiological role of this inhibitor in the regulation of the calcium pump in unknown.

## 3. The Transport Cycle.

Although it is now well documented that active calcium transport requires the splitting of ATP to ADP and $P_{i}$ (Schatmann, 1975; Sarkadi and Tosteson, 1979) the exact mechanism by which energy from the hydrolysis of ATP is utilized to transport calcium against an electrochemical gradient is not clearly understood. The reaction mechanism of the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase may be broken down into a series of steps or "partial reactions". Considerable progress has been made in the elucidation of the partial reactions of the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase in erythrocyte mem-
branes. The $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase reaction proceeds through a phosphorylated intermediate. The phosphorylated intermediate is a 135,000-145,000 dalton protein (Knauf et al, 1974; Katz and Blostein, 1975; Schatzmann and Burgin, 1978; Lichtner and Wolf, 1979; Carafoli et al, 1982) which is chemically distinct from the $\mathrm{Na}^{+}, \mathrm{K}^{+}$-ATPase phosphoenzyme of molecular weight around 100,000 daltons (Knauf et al, 1974; Katz and Blostein, 1975). Schatzmann (1985) recently presented a kinetic model, adapted from that proposed by Rega and Garrahan (1975), shown in Figure 1. This model incorporates most of the present knowlege concerning the reaction scheme of the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase.

The transport cycle is initiated when calcium and ATP interact at specific sites on the cytoplasmic face of the enzyme, resulting in formation of a phosphorylated intermediate of the enzyme. The enzyme demonstrates an absolute requirement for calcium at this step (Katz and Blostein, 1975; Rega and Garrahan, 1975; Schatzmann and Burgin, 1978; Niggli et al, 1979; Steiger and Luterbacher, 1981a). The calcium dependency of phosphorylation $\left(K_{d}=1-6 \mu M\right)$ is similar to that of the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase ( $\left.K_{d}=2-5 \mu M\right)$ (Knauf et al, 1974; Katz and Blostein, 1975; Rega and Garrahan, 1975; Richards et al, 1975). This form of the enzyme, exhibiting high affinity calcium binding sites oriented towards the cytoplasm, is commonly referred to as the $\mathrm{E}_{1}$ state. The phosphorylated enzyme complex, $E_{1} . C a . P$, which results from the transfer of the gamma phosphate group of ATP to a site on the enzyme, is sensitive to hydroxylamine and basic conditions (Katz and Blostein, 1975; Rega and Garrahan, 1975; Lichtner and Wolf,

Figure 1.
The reaction cycle of the human erythrocyte calcium pump. In the normal mode the cycle turns in a clock-wise direction. $\mathrm{Ca}_{\mathrm{i}}$ means ionized calcium in the cytosol, Ca o means ionized calcium in the extracellular medium and $P$ means inorganic phosphate. $E_{1}$ and $E_{2}$ are two conformational forms of the protein. Requirements of the reactions are indicated by asterisks. Taken from Schatzmann (1985).


1980b). These are properties associated with an acyl-phosphate bond, indicating that the phosphate group was transfered from ATP to a carboxylic acid group on the enzyme.

In the absence of calcium, magnesium will not initiate phosphorylation, however magnesium greatly accelerates formation of the phosphorylated intermediate (Rega and Garrahan, 1975; Garrahan and Rega, 1978). Schatzmann (1982) recently suggested that formation of the phosphoprotein may be a magnesium-dependent process, although there is evidence to the contrary in the literature (Rega and Garrahan, 1975). Monovalent cations, such as sodium and potassium, are also able to stimulate phosphorylation of the enzyme (Larocca et al, 1981).

Once formed, the intermediate complex $E_{1}$. Ca.P undergoes a conversion to a second state, $\mathrm{E}_{2}$.Ca.P. This step is thought to result in calcium translocation by a reorientation of the calcium-binding site towards the extracellular side of the membrane and a reduction in the affinity of this site for calcium by several orders of magnitiude (Sarkadi, 1980). Rega and Garrahan (1978) demonstrated that this step preceeded dephosphorylation and was stimulated by magnesium $\left(K_{d, ~} M g=8 \times 10^{-5} \mathrm{M}\right.$, Garrahan and Rega, 1978). In the absence of magnesium, transformation from the $E_{1}$.Ca.P state to the $E_{2}$.Ca.P state is believed to be very slow. Sarkadi (1980) has proposed that, in the absence of magnesium this step becomes rate-limiting. Lichtner and Wolf (1980a) have demonstrated that, analogous to calcium-dependent inhibition of $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase activity, excessive concentrations of calcium result in a large buildup of the phosphoprotein. This effect was attributed to inhibition of the conversion from
$\mathrm{E}_{1}$.Ca.P into $\mathrm{E}_{2} \cdot \mathrm{Ca} . \mathrm{P}$.
In the $E_{2}$ state, the phosphorylated intermediate is more reactive than in the $E_{1}$ state, and can undergo rapid hydrolysis. Rega and Garrahan (1975) suggested that when the enzyme is in the $\mathrm{E}_{2}$ state the phosphate group is more accessible to water, hence more readily hydrolysed. In a recent study, Chiesi and coworkers (1983) showed that the binding site for $P_{i}$ becomes less polar when the enzyme is in the $E_{2}$ state as compared to the $E_{1}$ state. This finding suggests that the water for hydrolysis may be displaced from another site within the binding pocket, rather than enter the pocket due to an increase in accessibility. In the model shown in Figure 1, Schatzmann (1985) has implied that the hydrolysis step is accelerated by both magnesium and ATP. Rega and Garrahan (1978) showed that, in the absence of magnesium, ATP could not stimulate decomposition of the phosphorylated intermediate. However, if the complex was formed in the presence of magnesium, and then the magnesium subsequently removed, ATP was able to accelerate decomposition of the phosphoprotein. These results suggest that magnesium was required at a step prior to dephosphoryation whereas free ATP stimulated the actual decomposition of the complex. The monovalent cations, sodium and potassium, are also able to stimulate dephosphorylation of the phosphorylated intermediate, either by promoting conversion of the intermediate complex into the $E_{2}$ state or by directly stimulating breakdown of the phosphoprotein (Enyedi et al, 1980b; Larocca et al, 1981). A very slow backward operation of the reaction cycle (ATP synthesis from $A D P$ and $P_{i}$ ) has been demon-
strated (Rossi et al, 1978; Wuthrich et al, 1979). hence, this reaction step is reversible. However, a very large calcium gradient is required, suggesting that reversal of dephosphorylation is thermodynamically unfavorable (Schatzmann, 1985).

The final step in the transport cycle is transition of the enzyme from the $E_{2}$ state back into the $E_{1}$ state. This allows the enzyme to begin a new reaction cycle. Although it was mentioned previously that magnesium stimulates formation of the $E_{1}$.Ca.P complex, Schatzmann (1985) has pointed out that the observed increase in the rate of phosphorylation could as easily be explained by accelerating the transition of the enzyme from $E_{2}$ back into the $E_{1}$ state. This proposed mechanism involves magnesium stimulation of the enzyme in a symmetrical fashion ( $E_{1}$ to $E_{2} ; E_{2}$ to $\left.E_{1}\right)$, however, it assumes that the equilibrium between $E_{2}$ and $E_{1}$ favors the $E_{2}$ state.

The sites of the regulatory effects of calmodulin on the calcium pump transport cycle include both phosphorylation (Enyedi et al, 1980; Muallem and Karlish, 1980; Rega and Garrahan, 1980) and dephosphorylation (Rega and Garrahan, 1980; Jeffery et al, 1981). However, there is very little known with regards to how these effects are produced.

Application of lanthanum to the external surface of erythrocytes inhibits calcium transport and $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase activity (Quist and Roufogalis, 1975; Szasz et al, 1978b). This treatment also causes accumulation of the phosphorylated intermediate (Szasz et al, 1978a; Schatzmann and Burgin, 1978). These results suggest that lanthanum is reacting with the phosphorylated intermediate while the enzyme is in the $E_{2}$ state. Lantha-
num will also stimulate phosphoenzyme formation in erythrocyte membrane fragments, in the absence of calcium (Szasz et al, 1978a). In addition, Szasz and coworkers (1978a) found that membrane fragments and inside-out vesicles were affected by lower concentrations of lanthanum than were intact erythrocytes. These results suggest a possible interaction with the $E_{1}$ state of the enzyme as well as the $E_{2}$ state, leading to increased phosphoprotein formation. Luterbacher and Shatzmann (1983) have suggested that lanthanum inhibits the conformational transformation of the phosphorylated enzyme from the $E_{1}$ state into the $E_{2}$ state. This mechanism does not explain how lanthanum acts from the extracellular surface, nor does it explain how the intermediate complex is formed in the absence of calcium. To explain the ability of lanthanum to inhibit the pump from the extracellular medium, Sarkadi (1980) has proposed that lanthanum binds to the phosphoprotein in the $E_{2}$ state following the dissociation of calcium, thereby stabilizing the enzyme in the phosphorylated form.

## IV Cystic Fibrosis

Cystic Fibrosis (CF) is a clinical disorder characterized by chronic obstructive lung disease, exocrine pancreatic insufficiency and elevated sweat electrolytes (di Sant' Agnese. and Talamo, 1967). Genetic evidence suggests tha CF is transmitted as an autosomal recessive trait (Lobeck, 1972; Sant' Agnese and Davis, 1976; Nader et al, 1978). The incidence of CF among white
populations is approximately 1 in 2000 live births (Nadler et al, 1978), while 1 in 20 individuals is a healthy carrier (Danks et al, 1965; Lobeck, 1972). The primary lesion(s) responsible for producing CF have not yet been identified.

Biochemical and histological evidence shows that all exocrine glands are affected in cystic fibrosis. As a rule, the secretions of exocrine glands in CF subjects behave in abnormal ways; they are thicker than normal (Wood et al, 1976), tending to precipitate and obstruct the ducts or passageways into which they are discharged. Sites of obstruction include lung airways, paranasal sinuses, mucus-secreting submandibular glands, small intestine, pancreas, biliary tract, and male and female reproductive tracts (Wood et al, 1976). This ducal obstruction can lead to fibrosis of certain exocrine glands or a deficiency of the exocrine secretion at its physiologic target organ. The end-results of this obstruction process include chronic pulmonary insufficiency, hepatic cirrhosis, pancreatic insufficiency, intestinal obstruction, mal-absorption and mal-digestion.

All patients who are homozygous for $C F$ also show a marked increase in the level of sodium, chloride and, to a lesser extent, potassium in their sweat (di Sant' Agnese et al, 1953). Morphologically, no difference has been seen between the eccrine sweat glands of patients with CF and those in control subjects (Munger et al, 1961). Using in vitro microperfusion, Mangos (1973) demonstrated that when normal sweat was perfused into CF sweat gland ducts, normal levels of sodium reabsorption were recorded, but when CF sweat was perfused into normal or CF sweat gland ducts, decreased levels of sodium reabsorption were obser-
ved. These results suggested that the abnormal electrolyte levels found in CF sweat are due to the presence of an inhibitory "factor" in CF sweat and not a defect in the ductal tissue.

Increased concentrations of sodium and chloride in sweat, without a parallel alteration in precursor fluid (Schultz, 1969) suggests that the plasma membrane transport of electrolytes by duct epithelium may be abnormal. Erythrocytes taken from CF patients have been used as a simple model system to examine plasma membrane transport of electrolytes as a possible defect. Balfe et al (1968) found that sodium transport was abnormal in erythrocytes from $C F$ patients who were either homozygotes or heterozygotes. These authors suggested that if the postulated defect of sodium transport observed in CF patients represents a primary defect in the disease state, a study of this abnormality in erythrocytes may help to determine the pathogenic effect. Subsequently, workers showed that only the ouabain-insensitive component of sodium efflux (Lapey and Gardner, 1971 ) and ATPase activity (Cole and Dirks, 1972) were decreased in erythrocytes from CF patients. These findings implied that an ATPase activity other than the one coupled to sodium transport was affected in the disease state. Horton et al (1970) measured the $\left(\mathrm{Ca}^{2+}+\right.$ $\mathrm{Mg}^{2+}$ )-ATPase activity of erythrocyte membranes from both normal subjects and CF patients and found a decreased activity in the erythrocytes from CF subjects. Recently, these results have been confirmed (Katz, 1978a; Ansah and Katz, 1980; Foder et al, 1980; Gietzen et al, 1984) and have been correlated with a decrease in calcium uptake into inside-out vesicles prepared from CF erythro-
cytes (Ansah and Katz, 1980). A similar reduction in ( $\mathrm{Ca}^{2+}+$ $\mathrm{Mg}^{2+}$ )-ATPase activity has been found in the plasma membranes of cultured skin fibroblasts from CF patients (Katz, 1978b; Katz and Ansah, 1980). Katz (1978a) reported that the decrease in $\left(\mathrm{Ca}^{2+}+\right.$ $\mathrm{Mg}^{2+}$ )-ATPase was due to a reduction in maximal activation and not a change in the affinity of the enzyme for calcium.

These studies do not indicate whether the reduction in plasma membrane $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase activity observed in CF subjects is a primary defect or whether it is a secondary effect due to some other in vivo or in vitro alteration in the plasma membrane of these cells. The defect could be in the structure of the calcium-transporting enzyme itself. Alternatively, the changes in $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase activity may reflect some regulatory or environmental modification which has occurred in vivo or was induced during in vitro handing of these CF cells. This second effect, namely an alteration induced during processing of the samples, may explain why several groups (Duffy et al, 1974; Feig et al, 1974; McEvoy et al, 1974) failed to observe any alteration in the activity of the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase in erythrocytes taken from CF patients. Recently, Miner et al (1983) observed a significant reduction in formation of the calciumdependent phosphorylated intermediate of the calcium pump in erythrocytes taken from $C F$ patients, suggesting a reduction in the number of active calcium-pumping sites per cell. Glaubensklee and Galey (1984) reported a $40 \%$ reduction in erythrocyte membrane associated immunoreactive calmodulin, suggesting a regulatory defect. However, addition of exogenous calmodulin to membrane fragments made from $C F$ erythrocytes did not return $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$

ATPase activity to control values (Katz and Emery, 1981; Gietzen et al, 1984). Other groups have shown differences in the lipid composition of CF erythrocyte membranes with respect to normal subjects (McEnvoy et al, 1974; Rogiers et al, 1980). Any of these changes could result in the deficiency in $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase activity observed in cystic fibrosis.

A recently reported property of the calcium-transport ATPase in erythrocyte membranes concerns its inhibition by calcium (Klinger et al, 1980; Vincenzi et al, 1980; Graf and Penniston, 1981), which is reportedly antagonized by magnesium (Klinger et al, 1980). Lichtner and Wolf (1980a) showed that excess calcium produced a buildup of the phosphorylated intermediate. To explain these results, Schatzmann (1982) has proposed that excess calcium (greater that $5.0 \times 10^{-5} \mathrm{M}$ ) inhibited the transformation of the intermediate complex from $E_{1}$. Ca.P to $E_{2} . C a . P$, by competing with magnesium at a "magnesium-specific" site, resulting in inhibition of dephosphorylation of the intermediate complex. The objective of the present study was to further characterize the inhibitory effect of calcium upon the transport cycle of the calcium transport ATPase. This required the determination of the effects of magnesium on the abibilty of calcium to inhibit dephosphorylation and the sensitivity of the phosphoprotein to dephosphorylation induced by either ATP or ADP.

Calmodulin, which is known to increase both the maximum velocity and calcium affinity of the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase, has also been reported to stimulate the rate of formation (Muallem and Karlish, 1980) and breakdown (Rega and Garrahan, 1980; Jeffery et al, 1981) of the phosphorylated intermediate of this enzyme. Jeffery et al (1981) found that magnesium and calmodulin both stimulated dephosphorylation on their own, however in the presence of magnesium ( 0.25 mM ) calmodulin had no additional stimulatory effects and vice versa. These results suggest that
these two activators may be acting at the same kinetic step. Magnesium is believed to enhance the rate of dephosphorylation by facilitating the transformation from $E_{1} \cdot C a . P$ to $E_{2} \cdot C a . P$ (Rega and Garrahan, 1975), hence this may also be the site at which calmodulin stimulates dephosphorylation. The effect of calmodulin on the rate of phosphoenzyme formation, reported by Muallem and Karlish (1980), could be explained by either a direct effect on the formation of the phosphoenzyme or by increasing the rate at which $E_{2}$ returns to the $E_{1}$ conformation following dephosphorylation. One aim of the present study was to further investigate the effects of calmodulin on the formation of the intermediate complex in order to determine the actual step at which calmodulin exerts its effects.

Recently, Schatzmann (1982) suggested that magnesium may be required for the first step in the transport cycle of the calcium pump, formation of a phosphorylated intermediate. Rega and Garrahan (1975) previously showed that phosphorylation of the enzyme did not require magnesium, however it is possible that there was sufficient magnesium contamination in their preparation to satisfy any magnesium requirements for phosphorylation. In the present study the magnesium chelator CDTA was employed to bind any remaining endogenous magnesium and establish whether or not there is in fact a magnesium requirement at this stage of the reaction cycle.

Decreased activity of the plasma membrane calcium transporting ATPase has been found in association with cystic fibrosis. Previous reports have revealed a specific decrease in
the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase activity of erythrocyte membrane preparations (Katz, 1978a). This finding has been supported by reports of a similar reduction in calcium uptake into inside-out vesicles prepared from the erythrocytes of subjects with cystic fibrosis (Ansah and Katz, 1980). Recently, Miner and coworkers (1983) demonstrated that, in the presence of lanthanum, phosphoprotein formation was less in the erythrocyte membranes from subjects with cystic fibrosis than those of normal subjects. It remained to be determined whether or not this change was due to a change in the enzyme itself versus a change in the number of active pumping sites present. This question was therefore investigated in this study.

## MATERIALS AND METHODS

## I. Materials

The chemicals and/or proteins were purchased from the following sources:

1. Sigma Chemical Co.

EGTA
EDTA
CDTA
Tris-ATP
2-Mercaptoethanol
Tris base
Tris hydrochloride
Lithium dodecylsulphate
Bovine serum albumin
Trichloroacetic acid
Hydroxylamine monohydrochloride
Bromophenol blue
Folin and Ciocalteu's phenol reagent
Glycerol
HEPES
Potassium chloride (KCl)
Magnesium chloride $\left(\mathrm{MgCl}_{2}\right)$
Sodium carbonate
Sucrose
TEMED
Citric acid

Tris-ADP
Potassium hydroxide (KOH)
Potassium phosphate $\left(\mathrm{KH}_{2} \mathrm{PO}_{4}\right)$
Activated charcoal (Norit-A)
Phosphoric acid
Citric acid (monohydrate)
Sodium citrate
Adenine hydrochloride
2. Amersham
[gamma- $\left.{ }^{32} \mathrm{P}\right]-$ ATP (Specific activity $\left.=20-40 \mathrm{Ci} / \mathrm{mmol}\right)$
3. ICN
$\left[2,8-{ }^{3} \mathrm{H}\right]-$ ATP $($ Specific activity $=20-40 \mathrm{Ci} / \mathrm{mmol})$
4. Calbiochem

Calmodulin (Bovine brain)
5. BDH Biochemicals

Calcium chloride $\left(\mathrm{CaCl}_{2}\right)$
Sodium dodecylsulphate
6. Bio-Rad

Acrylamide
N,N'-methylene-bisacrylamide
Coomassie Brilliant Blue R-250
Ammonium persulphate

# SDS-PAGE High Molecular Weight Standards SDS-PAGE Low Molecular Weight Standards 

7. Fisher Scientific Co. Sodium chloride (NaCl)

Lanthanum chloride ( $\mathrm{LaCl}_{3}$ )
Glacial Acetic acid
Cello-Seal
8. New England Nuclear

Aquasol liquid scintilation fluid

## II. Methods

1. Preparation of Calmodulin-Depleted Erythrocyte Membrane Fragments

Human blood, not more than four days old, was either obtained from the Red Cross or collected into EDTA-vacutainers and stored overnight before use. Erythrocytes were collected by centrifugation at 2500 g for 5 minutes and red cell membranes prepared by the method of Carafoli et al (1980) with some modifications: Erythrocytes were washed three times in five volumes of 130 mM KCl and 20 mM tris-Cl pH 7.4. The cells were then hemolysed in five volumes of 1 mM EDTA, 10 mM tris-Cl pH 7.4 with stirring for 10 minutes on ice followed by centrifugation at $18,000 \mathrm{~g}$ and $4^{\circ} \mathrm{C}$ for 10 minutes. This step was repeated five more times after which the membranes were washed five times in 10 mM K-HEPES, pH 7.4. The ghosts were resuspended in a storage buffer containing 20 mM K-HEPES $\mathrm{pH} 7.4,130 \mathrm{mM} \mathrm{KCl}, 0.5 \mathrm{mM} \mathrm{MgCl} \mathbf{2}^{\prime}$, 0.05 mM CaCl 2 and 2 mM dithiothreitol. The membrane suspension was quick-frozen in liquid nitrogen and stored for up to 5 days at $-80^{\circ} \mathrm{C}$.

Immediately before use, the membranes were thawed out, resuspended and washed three times in a solution containing 40 mm K-HEPES pH 7.4 at $10^{\circ} \mathrm{C}$.
2. Measurement of $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase Activity
$\mathrm{Ca}^{2+}$-ATPase activity was measured by a modification of the
method of Katz and Blostein (1975) in 0.2 ml of a medium containing (final concentration) $2 \mu \mathrm{M}$ tris-ATP (containing [gamma${ }^{32} \mathrm{PJ}$-ATP; $\left.0.056 \mathrm{Ci} / \mathrm{mmol}\right), 0.1 \mathrm{mM}$ EGTA, 40 mM K-HEPES $\mathrm{pH} 7.4,0.05$ $m M$ ouabain and the desired free calcium and magnesium concentrations. Calmodulin, lanthanum chloride, and/or CDTA were added to this medium when required. After preincubation for five minutes at $10^{\circ} \mathrm{C}$, the assay was started by the addition of 0.1 ml of membrane suspension (1-2 $\mathrm{mg} / \mathrm{ml})$. In experiments where determination of phosphoprotein formation and ATPase activity were performed simultaneously, a more concentrated membrane suspension was used ( $2-4 \mathrm{mg} / \mathrm{ml}$ ). After ten minutes the reaction was terminated by the rapid addition of 0.4 ml of an ice-cold stop solution containing $5 \%$ trichloroacetic acid (w/v), $2.5 \mathrm{mM} \operatorname{ATP}$ (disodium salt) and 5 mm potassium phosphate. The membranes were then pelleted by centrifugation at 1500 g and $4^{\circ} \mathrm{C}$ for 10 minutes and 0.3 ml of the supernatant was combined with 0.6 ml of charcoal suspension ( 0.19 g of Fisher Norit-A/ml 5\% TCA). After five minutes of vigorous mixing the samples were centrifuged for five minutes at 3000 g and 0.45 ml of the clear supernatant transfered to counting vials, 5 ml of Aquasol added and mixed. The vials were counted for 32 and the amount of ATP hydrolysis present expressed as pmol ATP hydrolysed per mg protein per minute as determined by the following equations:
specific activity $=\frac{\text { (media counts - background) }}{\text { total ATP }}$

ATPase $=\left(\operatorname{specif} \frac{(\text { sample counts }- \text { blank) (dil'n factor) }}{\text { ictivity) (rxn vol) (rxn time) (protein conc) }}\right.$
where:
specific activity $={ }^{32} \mathrm{P}$ counts (dpm) per pmol ATP. media counts $=32 \mathrm{p}$ counts $(\mathrm{dpm})$ present in 0.02 ml of media. total ATP $=$ total amount of ATP present in the incubation medium (400 pmol).
background $={ }^{32} \mathrm{P}$ counts $(\mathrm{dpm})$ in fluor alone. sample counts $={ }^{32} \mathrm{P}$ counts ( dpm ) obtained per sample. blank $=32 \mathrm{p}$ counts $(\mathrm{dpm})$ obtained in the absence of membranes. dilution factor $=$ correction for reaction volume sampled.
reaction volume $=$ final volume of reaction medium ( 0.2 ml ). reaction time $=$ period of enzyme-catalyzed hydrolysis. protein conc. = concentration (mg/ml) of erythrocyte membrane protein present in the reaction medium.
$\mathrm{Ca}^{2+}$-dependent ATPase activity was determined by subtracting the activity obtained in the presence of EGTA alone from that obtained when calcium was added to the reaction medium.

## 3. Phosphorylation of Erythrocyte Membranes

Phosphorylation of the membranes was performed at $10^{\circ} \mathrm{C}$ in 0.2 ml of a medium consisting of (final concentration) 40 mM K HEPES pH 7.4, $2 \mu \mathrm{M}$ tris-ATP (containing [gamma- ${ }^{32} \mathrm{P}$ ]-ATP, final activity $1.13 \mathrm{Ci} / \mathrm{mmol}$; $\left[2,8\right.$ - $\left.^{3} \mathrm{H}\right]-$ ATP, final activity 1.13 Ci/mmol), 0.1 mM tris-EGTA, 0.05 mM ouabain, and the desired free calcium and magnesium concentrations. Calmodulin, lanthanum chloride and/or CDTA were added to this medium when required. After five minutes of preincubation at $10^{\circ} \mathrm{C}$ the assay was started
by the addition of 0.1 ml of membrane suspension (2-4 $\mathrm{mg} / \mathrm{ml})$. In experiments where phosphoprotein formation was determined at various time points, the membrane suspension contained all reagents, except ATP, at their final concentrations. The reaction was terminated after various time intervals by the rapid addition of 0.4 ml of an ice-cold solution containing 5\% trichloroacetic acid (w/v), 2.5 mM ATP (disodium salt) and 5 mm potassium phosphate. The membranes were then pelleted by centrifugation at 1500 g and $4^{\circ} \mathrm{C}$ for 10 minutes. The supernatant was decanted and the pellet applied onto glass micro-fiber filters (Whatman GF/A), washed extensively with an ice-cold solution containing $5 \%$ trichloroacetic acid (w/v), air dried and counted for ${ }^{32} \mathrm{p}$ and ${ }^{3} \mathrm{H}$. The amount of phosphoprotein formed, expressed as fmol per $m g$ protein, was determined by the following calculations.
specific activity $=\frac{(\text { media counts }- \text { background) }}{\text { total ATP }}$
phosphoprotein $=\left(\operatorname{speci} \frac{1}{\text { fic activity })(\text { reaction vol })(\text { protein conc })} \quad-\right.$ (specific activity)(reaction vol)(protein conc)
where:
specific activity $={ }^{32} \mathrm{P} /{ }^{3} \mathrm{H}$ (dpm) counts per pmol ATP. media counts $=32 \mathrm{P} /{ }^{3} \mathrm{H}$ counts (dmp) present in $20 \mu \mathrm{l}$ of media. total ATP $=$ total amount of ATP present in the medium ( 400 pmol). background $={ }^{32} \mathrm{P} /{ }^{3} \mathrm{H}$ counts $(\mathrm{dpm})$ in fluor alone. sample counts $={ }^{32} \mathrm{P} /{ }^{3} \mathrm{H}$ counts $(\mathrm{dpm})$ obtained in sample.
reaction volume $=$ final volume of the reaction medium ( 0.2 ml ). protein concentration $=$ concentration ( $\mathrm{mg} / \mathrm{ml}$ ) of red cell membrane protein present in the reaction medium.
a. Dephosphorylation Experiments. Erythrocyte membrane fragments (1-2 $\mathrm{mg} / \mathrm{ml}$ ) were incubated as described previously, for 15 seconds at $10^{\circ} \mathrm{C}$ in phosphorylation medium containing the desired cations, then $20 \mu \mathrm{l}$ of a solution containing either 11 mm ADP or 11 mM cold ATP was added. After either 5 or 15 seconds the reaction was terminated by rapid addition of 0.4 ml of an ice cold solution containing $5 \%$ trichloroacetic acid (w/v), 2.5 mM ATP (disodium salt) and 5 mm potassium phosphate. The membranes were pelleted, washed and counted as described above. Total phosphorylation was that amount of phosphoprotein formed after a 15 second reaction time. The fraction of the total phosphoprotein remaining after the 'chase' was determined separately for each experiment then the mean and the standard error of the mean was calculated for each condition.
b. Hydroxylamine Treatment: Erythrocyte membrane fragments were incubated for 15 seconds at $10^{\circ} \mathrm{C}$ in phosphorylation medium containing the desired cations, as described previously, and then the reaction stopped by adding 0.4 ml of an ice-cold solution containing $15 \%$ trichloroacetic acid (w/v). The membranes were then pelleted by centrifugation at 1500 g and $4^{\circ} \mathrm{C}$ for 10 minutes and the supernatant decanted. Membranes were resuspended in 0.5 ml of either 0.6 M hydroxylamine/ 0.8 M sodium acetate pH 5.2 or
0.6 M sodium chloride/ 0.8 M sodium acetate pH 5.2 (control). After 10 minutes at room temperature, 2 ml of ice-cold $15 \%$ trichloroacetic acid (w/v) was added to the sample and the membranes pelleted by centrifugation at 1500 g and $4^{\circ} \mathrm{C}$ for 10 minutes, the supernatant removed and the pellet resuspended in either $5 \%$ trichloroacetic acid (for filtration) or sample buffer (for acid LiDS-PAGE).
4. Polyacrylamide Gel Electrophoresis and Autoradiography of Phosphorylated Erythrocyte Membrane Fragments
a. Polyacrylamide-Gradient Acid-Gel Electrophoresis. Polyacrylamide gradient (5-15\%) gels of 1.5 mm thickness were cast according to the method of Laemmli and Favre (1973). Buffers and running conditions which would stabilize the acyl-phosphate intermediate of the erythrocyte membrane $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase were modified from those described by Lichtner and wolfe (1979). The $5 \%$ "resolving" gel consists of 2.5 ml of an acrylamide-methylenebisacrylamide (30\%: 0.8\%) mixture, 0.1 M tris-phosphate buffer pH 7.0, $1.35 \%$ glycerol, $0.2 \%$ lithium dodecylsulphate (LiDS), $0.025 \%$ tetraethyl-methylenediamine (TEMED) and 0.3 ml ammonium persulphate $(7.5 \mathrm{mg} / \mathrm{ml})$ in a total volume of 15 ml . The $15 \%$ "resolving" gel contained 7.5 ml of acrylamide-methylene-bisacrylamide (30\%: 0.8\%), 0.1 M tris-phosphate buffer pH 7.0 , $5.75 \%$ glycerol, $0.2 \%$ LiDS, $0.05 \%$ TEMED and 0.225 ml ammonium persulphate $(7.5 \mathrm{mg} / \mathrm{ml})$ in a total volume of 15 ml . The $5 \%$ and the $15 \%$ "resolving" gel solutions were immediatly added to the gel chamber with the aid of a gradient former. About 0.5 ml of
distilled water was carefully layered on top of the gel and the gel was allowed to polymerize for 3-4 hours at room temperature. The distilled water was decanted prior to the addition of the "stacking" gel.

The "stacking" gel (3.5\%) consisted of 0.855 ml of acryl-amide-methylene-bisacrylamide (30\%: 0.8\%) mixture, 0.125 mM trisphosphate buffer $\mathrm{pH} 6.5,0.2 \%$ LiDS, $0.136 \%$ TEMED and 0.2 ml ammonium persulphate ( $15 \mathrm{mg} / \mathrm{ml}$ ) in a total volume of 7.33 ml . After mixing, the 'stacking' gel solution was gently poured into the gel chamber over the polymerized 'resolving' gel. A teflon 'comb' was then inserted into the stacking gel and the gel allowed to polymerize for at least 2 hours.

The gel buffer contained 20 mM tris-phosphate pH 7.0 and $0.2 \%$ LiDS.

Gels were run at $4^{\circ} \mathrm{C}$ under constant current ( $40 \mathrm{~mA} / \mathrm{slab}$ ) for 7 hours. The protein standards used for estimation of molecular weight (in daltons) were; myosin (200,000), Bgalactosidase $(116,250)$, phosphorylase $\mathrm{b}(92,500)$, bovine serum albumin $(66,200)$, ovalbumin $(45,000)$, carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme $(14,400)$.
b. Sample Preparation. When phosphorylated erythrocyte membranes were to be analysed on an acid gel the the reaction volume was reduced to $75 \mu \mathrm{l}, 3^{3} \mathrm{H}-\mathrm{ATP}$ was omitted from the medium the specific activity of the ${ }^{32} \mathrm{P}-\mathrm{ATP}$ was increased to 6.01 Ci/mmol (final activity) and the reaction was stopped with 0.4 ml
of an ice-cold solution containing $15 \%$ trichloroacetic acid (w/v). After stopping the reaction, the samples were centrifuged at 1500 g and $4^{\circ} \mathrm{C}$ for 10 minutes and the supernatant removed. The pellet was then resuspended in $25 \mu l$ of sample buffer containing (final concentration); 50 mM tris-phosphate pH 6.5 , $2.5 \%$ LidS, 0.12 M sucrose, 0.5 M 2 -mercaptoethanol and $0.01 \%$ bromophenol blue. Samples were heated at $60^{\circ} \mathrm{C}$ for 10 seconds, the pH neutralized with 2 M tris-base and $50 \mu \mathrm{of}$ each sample was applied per well.
c. Staining, Destaining and Autoradiography. The gels were stained with $0.25 \%$ Coomassie Brilliant Blue $\mathrm{R}-250$ in methanol/acetic acid/water (5:1:4) for 30 minutes at room temperature, followed by a first destaining procedure in methanol/acetic acid/water (45:1:15) for 1 hour ( 3 changes) and a second destaining procedure in methanol/acetic acid/water (4:1:15) until the background became transparent.

Prior to drying, the gels were fixed in acetic acid/glycerol/water (10:3:87) for 30 minutes, then $70 \%$ methanol for 20 minutes. The gels were immediatly dried under vacuum at $80^{\circ} \mathrm{C}$ for 90 minutes. The dried gels were exposed to X-ray film (Kodak Min-R) along with an intensifying screen (Cronex Lightning Plus, Dupont) for $7-10$ days at $-80^{\circ} \mathrm{C}$ following which the films were developed.
5. Miscellaneous Methods
a. Protein Assay. Erythrocyte membranes (10-80 $\mu \mathrm{g}$ ) were
assayed for protein using the standard protein assay of Lowry et al (1951). Bovine serum albumin was used as the standard protein.
b. Non-linear Analysis. Data was analysed using a FORTRAN non-linear reagression package 'NON-LIN' which fit the data to pre-chosen kinetic models. The models chosen were;
i. For ATPase data:

Model 1

$$
V_{\text {obs }}=(V \max )(S) /\left(S+K_{1}+s^{2} / K_{2}\right)
$$

Model 2
$V_{\text {obs }}=($ Vmax $)(S) /\left(S+K_{1}\right)$
where;
$V_{\text {obs }}=$ observed reaction velocity.
$\mathrm{V}_{\text {max }}=$ maximum reaction velocity.
$S=$ substrate (free calcium) concentration.
$K_{1}=f i r s t$ dissociation constant for the substrate.
$K_{2}=$ second dissociation constant for the substrate.
ii. For Phosphorylation data:
$\mathrm{v}_{\text {obs }}=\left(\mathrm{V}_{1}\right)(\mathrm{S}) /\left(\mathrm{S}+\mathrm{K}_{1}\right)+\left(\mathrm{V}_{2}\right)(\mathrm{S}) /\left(\mathrm{S}+\mathrm{K}_{2}\right)$
where:
$\mathrm{V}_{\text {obs }}=$ observed phosphoprotein formation.
$\mathrm{V}_{1}=$ maximum E.P formation for the first component.
$S=$ substrate (free calcium) concentration.
$K_{1}=$ first dissociation constant for the substrate.
$\mathrm{V}_{2}=$ maximum E.P formation for the second component.
$K_{2}=$ second dissociation constant for the substrate.
c. Statistical Analysis. Statistical analysis of all data was performed using a general least squares analysis of variance package (UBC:GENLIN). The variability in the mean values was expressed as the standard error of the mean (S.E.M.). Where mean values representing two or more experimental conditions were to be compared, the null hypothesis (no difference) was adopted. In order to test this hypothesis, a Newman-Keuls multiple range test was used to determine confidence intervals, at both the 0.05 and the 0.01 significance levels, for each sample mean. If two means being tested were found to occupy the same confidence interval, the null hypothesis was accepted. However, if the means did not occupy the same confidence interval the null hypothesis was rejected and the means values were said to be significantly different at the given significance level. Inter-assay variability was compensated for by performing an analysis of covariance.
d. Determination of Free Calcium Concentration. Free calcium concentrations were determined using the Fortran program 'CATIONS' written by Goldstein (1979). Equilibrium constants for cations and ligands were obtained fron Martell and Smith (1979) and were corrected for ionic strength, pH and temperature according to the methods described by these authors. Equilibrium constants for monoprotonated species were calculated according to the procedure of Blinks et al (1982).

## RESULTS

1. Effect of Calcium, Magnesium, Calmodulin and Lanthanum on Calcium-Dependent ATP Hydrolysis and Phosphoprotein Formation.

In these studies, calmodulin-depleted erythrocyte membranes were prepared by the method of Carafoli et al (1980), stored at $-80^{\circ} \mathrm{C}$ and used within 5 days. Unless stated otherwise assays of $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase activity and calcium-dependent phosphoprotein (E.Ca.P) formation were carried out at $10^{\circ} \mathrm{C}$ in the presence of $40 \mathrm{mM} \mathrm{K}-\mathrm{HEPES} \mathrm{pH} 7.4,25 \mu \mathrm{M} \mathrm{MgCl} 2$ and $2 \mu \mathrm{M}$ ATP, to reduce the turnover of the enzyme to facilitate determination of E.Ca.P and to minimize kinase-mediated phosphorylation.
a. Effect of Calcium Concentration. Figure 2 shows calcium dependent ATP hydrolysis and E.Ca.P formation as a function of free calcium $\left(\mathrm{Ca}^{2+}\right)$ concentration. The effect of $\mathrm{Ca}^{2+}$ concentration on ATPase activity was two-fold; it stimulated hydrolysis at low concentrations but inhibited activity at concentrations greater than $4-6 \times 10^{-5} \mathrm{M}$. Maximum velocity, from the mean of a number of experiments, under the assay conditions used was $49.4 \pm$ 3.7 pmol/mg/min (Table 1). Analysis of the activation curve by non-linear regression, as described in Methods, revealed that $\mathrm{Ca}^{2+}$ stimulation of $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase activity, in these cal-modulin-depleted membranes, was characterized by a dissociation constant $\left(K_{1}\right)$ of $4.24 \times 10^{-7} \mathrm{M}$ and calcium-dependent inhibition of the enzyme was characterized by a dissociation constant ( $\mathrm{K}_{2}$ ) of $1.68 \times 10^{-3} \mathrm{M}$ (Table 1). When determining phosphoprotein

Figure 2.
Effect of calcium concentration on ( $\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}$ )-ATPase activity and calcium-dependent phosphoprotein formation in erythrocyte membrane fragments at various calcium concentrations. ATPase activity ( - ) and phosphoprotein formation $(\bullet \longrightarrow)$ were determined as described in Methods. ATPase and phosphoprotein refer to the calcium component after subtraction of the values measured in the presence of EGTA ( 0.1 mM ) alone. The results shown here are the mean $\pm$ S.E.M. of observations from at least five membrane preparations with each membrane preparation assayed in triplicate.


Table 1

Kinetic parameters of the effect of calcium and calmodulin on $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase activity and the formation of the phosphorylated intermediate of the calcium pump from human erythrocyte membrane fragments.

1. ATPase activity:

$$
\begin{gathered}
\text { control } \\
(\mathrm{n}=6)^{\mathrm{a}}
\end{gathered} \quad+\underset{(\mathrm{n}=6)}{\text { calmodulin }}
$$

$K_{1}\left(x \quad 10^{-7}\right.$
M) ${ }^{C}$
$4.24 \pm 0.98^{b}$
$0.84 \pm 0.25$
$K_{2}\left(x 10^{-3}\right.$
M) ${ }^{C}$
$1.68 \pm 0.31$
$0.72 \pm 0.18$
$\mathrm{V}_{\text {max }}(\mathrm{pmol} / \mathrm{mg} / \mathrm{min})^{\mathrm{C}}$
$49.4 \pm 3.7$
$93.9 \pm 6.0$
2. Phosphoprotein formation:

|  | control <br> $(\mathrm{n}=5)$ | calmodulin <br> $(\mathrm{n}=5)$ |
| :--- | :---: | :---: |
| $\mathrm{K}_{1}\left(\mathrm{x} 10^{-8} \mathrm{M}\right)^{d}$ | $5.06 \pm 1.51$ | $4.72 \pm 0.82$ |
| $\mathrm{~K}_{2}\left(\mathrm{x} 10^{-4} \mathrm{M}\right)^{\mathrm{d}}$ | $7.16 \pm 0.27$ | $8.89 \pm 0.82$ |
| $\mathrm{~V}_{1}(\mathrm{fmol} / \mathrm{mg})^{\mathrm{d}}$ | $400 \pm 18$ | $641 \pm 45$ |
| $\mathrm{~V}_{2}(\mathrm{fmol} / \mathrm{mg})^{\mathrm{d}}$ | $4020 \pm 210$ | $3835 \pm 221$ |

a 'n' refers to the number of membrane preparations assayed; assay was performed in triplicate.
b Results are expressed as the mean $\pm$ SEM.
c Kinetic constants were estimated by model 1 described in Methods
d Kinetic constants were estimated as described in Methods
formation as a function of $\mathrm{Ca}^{2+}$ concentration, a 15 second reaction time was chosen in order to allow measurement of the steadystate E.Ca.P levels while minimizing the contribution from kinase-mediated phosphorylation. As shown in Figure 2, the calcium-dependent E.Ca.P formation was biphasic, with a high calcium affinity component, $K_{1}=5.1 \times 10^{-8} \mathrm{M}$ and a low calcium affinity component, $K_{2}=7.2 \times 10^{-4} \mathrm{M}$ (Table 1 ). The high affinity component was associated with low levels of E.Ca.P and a very shallow response to changing calcium concentration, whereas in the region of the low affinity component E.Ca.P rose sharply to reach plateau levels which were 10 times higher than observed in the high affinity region of the calcium curve. The low affinity component of E.Ca.P and the calcium-dependent inhibition of $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase activity demonstrated very similar calcium dissociation constants, $7.2 \times 10^{-4} \mathrm{M}$ and $1.9 \times 10^{-3} \mathrm{M}$ (Table 1 ), respectively.

Autoradiograms of phosphorylated erythrocyte membranes, as shown in Figure 3, reveal that at all calcium concentrations tested the majority of the ${ }^{32} \mathrm{P}$ lable was incorporated into a protein with an apparent molecular weight of approximatly 140,000 daltons. Phosphorylation of this protein was calcium-dependent and sensitive to treatment with hydroxylamine. This confirmed that the high levels of phosphoprotein formed in the presence of millimolar concentrations of calcium did, in fact, represent the acyl-phosphate intermediate of the calcium pump (E.Ca.P) and not a kinase-mediated phosphorylation of other membrane proteins. The ratio of ATPase activity to E.Ca.P formation, referred

Figure 3.
Acid-Polyacrylamide gel electrophoresis and autoradiography of calcium dependent acyl-phosphate formation and hydroxylamine sensitivity. ${ }^{32} \mathrm{P}$ was incorporated at the indicated free calcium concentrations followed by treatment with either 0.6 M hydroxylaminel 0.8 M Na-acetate, $\mathrm{pH} 5.2(+)$ or $0.6 \mathrm{M} \mathrm{NaCl} / 0.8 \mathrm{M} \mathrm{Na-}$ acetate, $\mathrm{pH} 5.2(-) . \quad$ Proteins were denatured with LiDS then separation on a 5-15\% acrylamide-gradient slab gel. Phosphorylation, hydroxylamine treatment, electrophoresis, autoradiography, and determination of free calcium concentrations were as described in Methods.

to as the turnover number of the enzyme, is an index of the rate at which the individual pump molecules are cycling under the various assay conditions utilized. Figure 4 shows that turnover of the enzyme was highest at calcium concentrations of $1-5 \times 10^{-6}$ M. At this calcium concentration ATPase activity was reaching maximum velocity whereas very little E.Ca.P was accumulated. To study the effect of free calcium concentration on the rate of formation and steady-state level of E.Ca.P two calcium concentrations were chosen for further investigation from the curve of ATPase activity versus calcium concentration shown in Figure 2. The free calcium values chosen were $1.0 \times 10^{-6} \mathrm{M}$ and $4.0 \times 10^{-4} \mathrm{M}$. At both of these calcium concentrations ATPase activity was approximatly $70-80 \%$ of maximum velocity, however 1.0 $x \quad 10^{-6} \mathrm{M} \mathrm{Ca}^{2+}$ is in the range where calcium activates ATPase activity whereas $4.0 \times 10^{-4} \mathrm{M} \mathrm{Ca}^{2+}$ is in the range of calciuminhibition. Determination of E.Ca.P formation over time in the presence of $1.0 \times 10^{-6} \mathrm{M}$ or $4.0 \times 10^{-4} \mathrm{M} \mathrm{Ca}^{2+}$, as shown in Figure 5 indicates that in the presence of $4.0 \times 10^{-4} \mathrm{M} \mathrm{Ca}^{2+}$, E.Ca.P reached steady-state levels within 15 to 20 seconds whereas in the presence of $1.0 \times 10^{-6} \mathrm{M} \mathrm{Ca}^{2+}$ steady state was not reached even after 30 seconds. Under these conditions ATPase activity was greater at the lower calcium concentration.

Lanthanum is a trivalent cation known to inhibit calcium transport (Quist and Roufogalis 1975, Sarkadi et al 1977) and calcium-dependent ATPase activity (Weiner and Lee 1972) in erythrocyte membranes. Several authors (Szasz et al 1978, Schatzmann and Burgin 1978, Muallem and Karlish 1982) have also found that lanthanum increased the steady-state level of phosphoprotein.

Figure 4.
Effect of calcium concentration on the turnover of the $\left(\mathrm{Ca}^{2+}+\right.$ $\mathrm{Mg}^{2+}$ )-ATPase in erythrocyte membrane fragments. The turnover number is the ratio of ATPase activity to phosphoprotein formation. The result shown is the mean $\pm$ S.E.M. of observations fron five membrane preparations; each membrane preparation was assayed in triplicate.


Figure 5.
Effect of calcium concentration on ( $\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}$ )-ATPase activity and the time course of calcium-dependent phosphoprotein formation in erythrocyte membrane fragments. ATPase activity and phosphoprotein formation were measured in the presence of either $1 \quad \mu \mathrm{M}$ $(\bullet)$ or $0.4 \mathrm{mM}(\longrightarrow)$ free calcium. ATPase activity and phosphoprotein formation were determined as described in Methods. The result shown is the mean $\pm$ S.E.M. of observations from five membrane preparations; each membrane preparation was assayed in triplicate. ATPase activity and phosphoprotein formation were determined in parrallel for each membrane preparation. Single asterisk indicates significantly different from control at $p<0.05$. Double asterisk indicates significantly different from control at $p<0.01$.


Lanthanum is now thought to inhibit dephosphorylation of the phosphorylated intermediate. As the rate of formation and steady state level of E.Ca.P observed in a time-course study actually represents the net effect of phosphorylation and dephosphorylation reactions, the effect of lanthanum on the time-course was studied in order to determine the rate of formation and steadystate level of phosphoprotein when its breakdown was inhibited. In this study, shown in Figure 6 , lanthanum ( 0.1 mm ) inhibited ATPase activity by $68 \%$ while it stimulated the rate of phosphoprotein formation and increased the steady-state level of phosphoprotein by $141 \%$. In a recent article Luterbacher and Schatzmann (1983) suggested that lanthanum blocked dephosphorylation by inhibiting the conformational change from $E_{1} . C a . P$ to E2.Ca.P. This theory was tested by examining the effect of lanthanum ( 0.2 mm ) on the amount of phosphoprotein formed in the presence of 10 mm calcium. The buildup of phosphoprotein in the presence of 10 mM calcium is also believed to be due to an inhibition of dephosphorylation either at this same step or at the subsequent step, by limiting the dissociation of calcium from the low affinity binding site on $\mathrm{E}_{2}$.Ca.P. In the presence of both lanthanum and calcium, phosphoprotein formation was reduced by $71 \%$ compared to calcium alone (Table 2). Thus, lanthanum appeared to compete with the ability of calcium to accumulate phosphoprotein.
b. Effect of Calmodulin. Calmodulin was added to cal-modulin-depleted erythrocyte membranes in order to study its

Figure 6.
Effect of lanthanum on $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase activity and the time course for calcium-dependent phosphoprotein formation in erythrocyte membrane fragments. ATPase activity and phosphoprotein formation, measured in the presence of either 0.15 mM total added $\mathrm{CaCl}_{2}(\bullet)$ (control) or 0.15 mM CaCl 2 plus $0.1 \mathrm{mM} \mathrm{LaCl}_{3}$ (m), were determined as described in Methods. The result shown is the mean $\pm$ S.E.M. of observations from four membrane preparations; each membrane preparation was assayed in triplicate. ATPase activity and phosphoprotein formation were determined simultaneously for each membrane preparation. Asterisk indicates significantly different from control, p<0.01.


Table 2.

The effect of $\mathrm{LaCl}_{3}$ on formation of the phosphorylated intermediate of the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase in the presence and absence of 10 mM CaCl 2 .

| Condition | Phosphoprotein formation (fmol/mg) | \% change |
| :---: | :---: | :---: |
| $0.2 \mathrm{mM} \mathrm{LaCl}_{3}$ | 429 | - |
| 10 mM CaCl 2 | 4462 | - |
| $\begin{aligned} & 0.2 \mathrm{mM} \mathrm{LaCl}_{3}+ \\ & 10 \mathrm{mM} \mathrm{CaCl} \end{aligned}$ | 1300 | -71\% |

Phosphoprotein formation was measured as described in methods. Results shown represent a single experiment.
effect on both the rate of formation and the steady-state levels of E.Ca.P. The effects of calmodulin on the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase in erythrocyte plasma membranes are well documented: they are, 1) an increase in the observed maximum velocity; and 2 ) an increase in the affinity of the enzyme for calcium. This is demonstrated in Figure 7, showing the effects of exogenously added calmodulin ( $0.01 \mathrm{mg} / \mathrm{ml}$ ) on ATPase activity at various $\mathrm{Ca}^{2+}$ concentrations. Calmodulin increased the $V_{\text {max }}$ for hydrolysis of ATP by $90 \%$ (from 49.4 to 93.9 pmol/mg/min), produced a small but not significant decrease in the dissociation constant for calcium (from $4 \times 10^{-7}$ to $0.8 \times 10^{-7} \mathrm{M}$; Table 1 ), but had no effect on the calciumdependent inhibition of ATPase activity. In contrast to its effects on ATPase activity, calmodulin addition had no significant effect on E.Ca.P levels when assayed at 15 seconds. In the presence of calcium concentrations ranging from $10^{-8}$ to $10^{-4}$ M, exogenously added calmodulin produced a small, but not significant increase in E.Ca.P levels (Table 1). However, at higher $\mathrm{Ca}^{2+}$ concentrations exogenous calmodulin had no effect on E.Ca.P (Table 1, Figure 8a). Analysis of this data by either nonlinear regression (Table 1) or by an Eadie-Hoffstee plot (Figure 8b) did not reveal any calmodulin-induced changes in either of the two calcium dissociation constants. Turnover of the enzyme (Figure 9) was increased by calmodulin at low calcium concentrations, however this effect was only significant at the lowest calcium concentration used (5.08 $\left.\times 10^{-8} \mathrm{M}\right)$.

To examine the effect of calmodulin on E.Ca.P formation as a function of time, two calcium concentrations were chosen from Figure 1 as in the previous section, one in the range of calcium

Figure 7.
Effect of calmodulin on $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase activity in calmo-dulin-depleted erythrocyte membrane fragments at various calcium concentrations. Calmodulin-stimulated (10 $\mu \mathrm{g} / \mathrm{ml})(\square)$ and basal $(\bullet \rightarrow)$ ATPase activity was determined as described in Methods. ATPase refers to the calcium component of hydrolysis after subtraction of the values measured in the presence of EGTA (0.1 mM) alone. The result shown is the mean $\pm$ S.E.M. of observations from six membrane preparations; each membrane preparation was assayed in triplicate. Asterisk indicates significantly different from basal activity, p < 0.01.


Figure 8 a and $b$.
Effect of calmodulin on calcium-dependnet phosphoprotein formation in calmodulin-depleted erythrocyte membrane fragments at various free calcium concentrations. Calmodulin-stimulated (10 $\mu \mathrm{g} / \mathrm{ml})(\longrightarrow)$ and basal $(\bullet \longrightarrow)$ phosphoprotein formation were determined as described in Methods. Phosphoprotein refers to the calcium component of phosphorylation after subtraction of the values measured in the presence of EGTA ( 0.1 mM ) alone. The result shown is the mean $\pm$ S.E.M. of observations from five membrane preparations; each membrane preparation was assayed in triplicate. Figure $8 b$ is an Eadie-Hoffstee plot of the same data.



Figure 9.
Turnover of the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase in erythrocyte membrane fragments in the absence $(\bullet \longrightarrow)$ (control) and presence ( of exogenous calmodulin ( $10 \mu \mathrm{~g} / \mathrm{ml}$ ) at various free calcium concentrations. The turnover number is the ratio of ATPase activity to phosphoprotein formation. Free calcium concentrations were determined as described in Methods. The result shown is the mean $\pm$ S.E.M. of observations from five membrane preparations; each membrane preparation was assayed in triplicate. Asterisk indicates significantly different from control, $\mathrm{p}<0.01$.

activation of the ATPase ( $1.0 \times 10^{-6} \mathrm{M}$ ) and the other in the range of calcium-dependent inhibition of ATPase activity $\left(4.0 \times 10^{-4} \mathrm{M}\right)$. In the presence of $1.0 \times 10^{-6} \mathrm{M} \mathrm{Ca}^{2+}$ (Figure 10), calmodulin stimulated ATPase activity by $64 \%$ and increased the rate of formation of E.Ca.P and its level at all time points up to 30 seconds. Under these conditions steady-state was not reached. When the calcium concentration was increased to 4.0 x $10^{-4} \mathrm{M}$ (Figure 11), calmodulin enhanced ( $\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}$ )-ATPase activity by 48\%; it did not effect the rate of formation of E.Ca.P but it reduced the steady-state for E.Ca.P by $27 \%$. As discussed in the previous section, lanthanum is thought to block dephosphorylation of the phosphorylated intermediate. In order to observe the effect of calmodulin on the formation of the phosphoprotein with minimal interference from dephosphorylation, lanthanum ( 0.1 mm ) was added to the reaction medium. In the presence of $0.1 \mathrm{M} \mathrm{LaCl}_{3}$ and $0.15 \mathrm{mM} \mathrm{CaCl}{ }_{2}$ (Figure 12), calmodulin stimulated ATP hydrolysis and it increased the apparent rate of formation and steady-state level of E.Ca.P.
c. Effects of Magnesium. In order to determine if magnesium is a pre-requisite for formation of the ternary complex $E_{1}$.Ca.P, the magnesium chelator CDTA was added to the assay medium in place of magnesium. Figure 13 shows $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase activity, at various free calcium concentrations, in the presence of either $0.025 \mathrm{mM} \mathrm{Mg}^{2+}$ (the standard assay condition) or 0.1 mM CDTA. In the absence of free magnesium, the $\mathrm{V}_{\text {max }}$ for ATP hydrolysis was reduced from 25.0 to $17.4 \mathrm{pmol} / \mathrm{mg} / \mathrm{min}$ and the

Figure 10.
Effect of calmodulin on ( $\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}$ )-ATPase activity and the time course of calcium-dependent phosphoprotein formation in erythrocyte membrane fragments in the presence of $1 \quad \mu \mathrm{M}$ free calcium. Calmodulin-stimulated (10 $\mu \mathrm{g} / \mathrm{ml})(\square)$ and basal $(\bullet)$ ATPase activity and phosphoprotein formation were determined as described in Methods. The result shown is the mean $\pm$ S.E.M. of five membrane preparations; each membrane preparation was assayed in triplicate. ATPase activity and phosphoprotein formation were determined simultaneously for each membrane preparation. Asterisk indicates significantly different from basal activity, $\mathrm{p}<0.01$.


Figure 11.
Effect of calmodulin on ( $\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}$ )-ATPase activity and the time course of calcium-dependent phosphoprotein formation in erythrocyte membrane fragments in the presence of 0.4 mM free calcium. Calmodulin-stimulated ( $10 \quad \mu \mathrm{~g} / \mathrm{ml}$ ) (m-m) and basal $(\bullet)$ ATPase activity and phosphoprotein formation were determined as described in Methods. The result shown is the mean $\pm$ S.E.M. of observations from five membrane preparations; each membrane preparation was assayed in triplicate. ATPase activity and phosphoprotein formation were determined simultaneously for each membrane preparation. Asterisk indicates significantly different from basal activity, p<0.01.


Figure 12.
Effect of calmodulin on ( $\left.\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase activity and the time course of calcium-dependent phosphoprotein formation in erythrocyte membrane fragments in the presence of lanthanum. Calmodulin-stimulated (10 $\mu \mathrm{g} / \mathrm{ml})(\longrightarrow)$ and basal ( $\longrightarrow$ ) ATPase activity and phosphoprotein formation, measured in the presence of 0.15 mM CaCl 2 and 0.1 mM LaCl 3 , were determined as described in Methods. The result shown is the mean $\pm$ S.E.M. of five membrane preparations; each membrane preparation was assayed in triplicate. ATPase activity and phosphoprotein formation were determined simultaneously for each membrane preparation. Single asterisk indicates significantly different from basal activity at $p<0.05$. Double asterisk indicates significantly different from basal activity at $p<0.01$.


Figure 13.
Effect of magnesium on ( $\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}$ )-ATPase activity in erythrocyte membrane fragments at various free calcium concentrations. ATPase activity was assayed in the presence of either $25 \mu \mathrm{M}$ magnesium (—) or 0.1 mM CDTA ( $-\longrightarrow$ ). ATPase activity was determined as described in Methods. ATPase refers to the calcium component of hydrolysis after subtraction of the values measured in the presence of EGTA ( 0.1 mm ) alone. The result shown is the mean $\pm$ S.E.M. of observations from five membrane preparations; each membrane preparation was assayed in triplicate.

$K_{d}$ of the enzyme for calcium was increased slighty from 0.60 x $10^{-6}$ to $3.1 \times 10^{-6} \mathrm{M}$ (Table 3). Phosphoprotein formation, shown in Figures 14 a and $b$, was unaffected by chelation of magnesium at all calcium concentrations from $5.0 \times 10^{-8}$ to $1.0 \times 10^{-2} \mathrm{M}$. Turnover of the enzyme (Figure 15) was considerably depressed with respect to the control condition. In a time-course study where the calcium concentration was $1.0 \times 10^{-6} \mathrm{M}$, chelation of magnesium produced a marked reduction in ATPase activity, but only a small depression in E.Ca.P levels at all time points studied (Figure 16). When the calcium concentration was increased to $4.0 \times 10^{-4} \mathrm{M}$, the rate of formation and the steady state level of $E . C a . P$ were unaffected by chelation of free magnesium, while $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase activity was only slightly depressed, as shown in Figure 17.

Micromolar concentrations of magnesium stimulate a conformational change of the enzyme from the high-energy $E_{1}$.Ca. P complex to the lower energy form $E_{2} \cdot C a . P$. The $E_{2} \cdot C a . P$ form of the enzyme demonstrates a lower affinity for calcium and a much higher reactivity towards water. In response to suggestions by several authors that the calcium-dependent inhibition of ( $\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}$ ) ATPase activity and concomitant buildup of phosphoprotein was due to calcium competing with magnesium at this site, thereby resulting in inhibition of the conversion of $E_{1}$.Ca.P to $E_{2}$.Ca.P, the low affinity component of phosphoprotein formation was studied in the presence of either 0.1 mM CDTA or $0.5 \mathrm{mM} \mathrm{Mg}{ }^{2+}$. Though formation of E.Ca.P was stimulated slightly by $0.5 \mathrm{mM} \mathrm{Mg}^{2+}$ at lower calcium concentrations (Figure 18), the buildup of phosphoprotein at higher calcium concentrations was not affected by magnesium

Table 3.
Kinetic parameters of the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase in the presence of either $25 \mu \mathrm{M} \mathrm{Mg}{ }^{2+}$ or 0.1 mM CDTA.

$$
\begin{array}{cc}
25 \mu \mathrm{M} \mathrm{Mg}^{2+} & 0.1 \mathrm{mM} \mathrm{CDTA} \\
(\mathrm{n}=5)^{\mathrm{a}} & (\mathrm{n}=5)
\end{array}
$$

$K_{m}\left(x 10^{-7}\right.$
M) ${ }^{C}$
$5.97 \pm 2.26^{b}$
$25.7 \pm 10.3$
$V_{\text {max }}(\mathrm{pmol} / \mathrm{mg} / \mathrm{min})^{\mathrm{C}}$
$25.0 \pm 3.61$
$17.4 \pm 2.9$
a 'n' refers to the number of membrane preparations assayed; each assay was performed in triplicate.
b Results are expressed as the mean $\pm$ SEM.

C Kinetic constants were estimated by model 2 as described in Methods.

Figure 14 a and $b$.
Effect of magnesium on calcium-dependent phosphoprotein formation in erythrocyte membrane fragments at various free calcium concentrations. Phosphoprotein formation was assayed in the presence of either $25 \mu \mathrm{M}$ magnesium ( $\quad$ ) or 0.1 mM CDTA ( $\longrightarrow$ ). ATPase activity was determined as described in Methods. Phosphoprotein refers to the calcium component of phosphorylation after subtraction of the values measured in the presence of EGTA ( 0.1 mm ) alone. The result shown is the mean $\pm$ S.E.M. of observations from four membrane preparations; each membrane preparation was assayed in triplicate.



Figure 15.
Turnover of the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase in erythrocyte membrane fragments in the presence of either $25 \mu \mathrm{M}$ magnesium ( $\quad$ ) or 0.1 mM CDTA $(\longrightarrow)$ at various free calcium concentrations. The turnover number is the ratio of ATPase activity to phosphoprotein formation. Free calcium concentrations were determined as described in Methods. The result shown was determined from the means of five ATPase assays and four phosphorylation assays.


Figure 16.
Effect of magnesium on $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase activity and the time course of calcium-dependent phosphoprotein formation in erythrocyte membrane fragments in the presence of $1.0 \mu \mathrm{M}$ free calcium. ATPase activity and phosphoprotein formation in the presence of either $25 \mu \mathrm{M}$ magnesium $(\longrightarrow)$ or 0.1 mM CDTA $(\backsim)$ were determined as described in Methods. The result shown is the mean $\pm$ S.E.M. of observations from four membrane preparations; each membrane preparation was assayed in triplicate. ATPase activity and phosphoprotein formation were determined simultaneously for each membrane preparation. Asterisk indicates significantly different from $25 \mu \mathrm{M}$ magnesium condition, $\mathrm{p}<0.01$.


Figure 17.
Effect of magnesium on $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase activity and the time course of calcium-dependent phosphoprotein formation in erythrocyte membrane fragments in the presence of 0.4 mM free calcium. ATPase activity and phosphoprotein formation in the presence of either $25 \mu \mathrm{M}$ magnesium $(\bullet)$ or 0.1 mM CDTA $(\longrightarrow)$ were determined as described in Methods. The result shown is the mean $\pm$ S.E.M. of observations from four membrane preparations; each membrane preparation was assayed in triplicate. ATPase activity and phosphoprotein formation were determined simultaneously for each membrane preparation.


Figure 18.
Effect of magnesium on the low calcium affinity component of calcium-dependent phosphoprotein formation. Phosphoprotein formation in the presence of either 0.5 mM magnesium (—) or 0.1 mM CDTA (—) were determined as described in Methods. Phosphoprotein refers to the calcium component after subtraction of the values measured in the presence of EGTA ( 0.1 mM ) alone. The result shown is the mean $\pm$ S.E.M. of observations from at least three membrane preparations; each membrane preparation was assayed in triplicate. The asterisk indicates the result is significantly different from the CDTA condition, $p<0.01$.

(Figure 18). In addition, E.Ca.P formation in the presence of 10 $\mathrm{mM} \mathrm{Ca}{ }^{2+}$ (Figure 19) was not affected by magnesium concentrations as high as 1 mM . Table 4 shows that E.Ca.P produced in the presence of $10 \mathrm{mM} \mathrm{Ca}{ }^{2+}$ was rapidly degraded in the presence of either 1 mM ADP or 1 mM cold ATP, and that concentrations of magnesium up to 1 mM had no significant effect on the sensitivity of E.Ca.P to hydrolysis by either ATP or ADP. Following a 5 second exposure to 1 mM ADP, $22 \%$ of the phosphoprotein remained, which was significantly less than the $39 \%$ which remained following 5 second exposure to ATP. After a 15 second exposure time however, there was no significant difference in residual E.Ca.P following ADP or ATP treatment. Therefore both ADP and ATP appear to dephosphorylate E.Ca.P, in a largely magnesiumindependent manner (Table 4).
II. ATP Hydrolysis and Phosphoprotein Formation in the Erythrocyte Plasma Membranes of Subjects with Cystic Fibrosis

The possible role of the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase in the defect in calcium handling associated with cystic fibrosis was further studied under conditions designed to accumulate phosphoprotein, by inhibiting dephosphorylation of the enzyme. Both 10 mM CaCl 2 and 0.1 mM LaCl 3 caused a large buildup in the phosphorylated intermediate such that $\left[E_{1}+E_{2}\right] \ll\left[E_{1} P+E_{2} P\right]$. This provided a means of comparing the number of functional enzyme units in erythrocyte plasma membrane from cystic fibrosis subjects compared to those from age- and sex-matched controls. In the pres-

Figure 19.
Effect of magnesium on phosphoprotein formation in the presence of 10 mM calcium. Phosphoprotein formation, determined as described in Methods, refers to total phosphorylation. The result shown is the mean $\pm$ S.E.M. of observations from at least three membrane preparations; each membrane preparation was assayed in triplicate.


## Table 4.

Effect of exposure to either 1 mM ADP or 1 mM cold ATP on the acyl-phosphate intermediate of the erythrocyte (Ca+Mg)-ATPase formed in the presence of $10 \mathrm{mM} \mathrm{Ca}{ }^{2+}$ and various concentrations of magnesium. Membrane fragments were phosphorylated for 15 seconds in the presence of various magnesium concentrations, then dephosphorylation was initiated by addition of either ADP or ATP (1 mM final concentration) and the reaction terminated after either 5 or 15 seconds by the rapid addition of ice cold 5\% TCA solution.

Percentage of the Total Phosphoprotein Remaining

|  | ADP |  | ATP |  |
| :---: | :---: | :---: | :---: | :---: |
|  | $\begin{aligned} & 5 \mathrm{sec} \\ & (n=3) \end{aligned}$ | $\begin{array}{r} 15 \mathrm{sec} \\ \quad(\mathrm{n}=4) \end{array}$ | $\begin{aligned} & 5 \mathrm{sec} \\ & (\mathrm{n}=3) \end{aligned}$ | $\begin{array}{r} 15 \mathrm{sec} \\ (\mathrm{n}=4) \end{array}$ |
| 0.1 mM CDTA | 25\% $\pm 3 \%$ | $9 \% \pm 3 \%$ | $38 \% \pm 3 \%^{*}$. | $11 \% \pm 5 \%^{* *}$ |
| $25 \mu \mathrm{Mmg}$ | $21 \% \pm 2 \%$ | $12 \% \pm 28$ | $36 \%$ 土 $3 \%^{*}$ | 16\% $\pm 4 \%^{* *}$ |
| 0.5 mM Mg | 18\% $\pm 2 \%$ | $9 \% \pm 2 \%$ | $39 \% \pm 4 \%^{*}$ | $9 \% \pm 1 \%^{* *}$ |
| 1.0 mM Mg | $24 \% \pm 2 \%$ | $18 \% \pm 4 \%$ | $45 \%$ 土 $7 \%$ * | $11 \% \pm 4 \%^{* *}$ |

note: the effects of magnesium were found to be non-significant. Signifies difference from 5 second treatment with ADP ( $\mathrm{p} \leq 0.01$ ) .
Signifies difference from 5 second treatment with ATP ( $\mathrm{p}<0.01$ ).
' $n$ ' refers to the number of membrane preparations assayed; each preparation was assayed in triplicate.
Results are expressed as the mean $\pm$ SEM.
ence of $10 \mathrm{mM} \mathrm{Ca}{ }^{2+}$ a $16 \%$ drop in the steady-state level of E.Ca.P was noted in CF compared to the control group (Table 5). ( $\mathrm{Ca}^{2+}+$ $\mathrm{Mg}^{2+}$ )-ATPase activity (Table 5) was also lower in CF membranes however there was no difference in the turnover number of the enzyme between the two groups. When 0.1 mM LaCl 3 was used there was less buildup of the steady-state level of phosphoprotein, however CF samples again had a significantly lower steady-state level of the phosphorylated enzyme and ATP hydrolysis with respect to control values (Table 5). Turnover numbers did not differ. Phosphoprotein formation and ATPase activity in the presence of 0.025 mM MgCl 2 alone (Table 5) were not altered in the $C F$ membranes.

Calcium-dependent phosphoprotein formation, $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase activity and turnover in cystic fibrosis (CF) and control erythrocyte membranes in the presence of $25 \mu \mathrm{M}$ magnesium and either 10 mM calcium chloride or 0.1 mM lanthanum chloride. ATPase and phosphoprotein refer to the components remaining, after subtraction of values measured in the presence of $25 \mathrm{uM} \mathrm{Mg}^{2+}$ and 0.1 mM EDTA (basal activity). Turnover is the ratio of ATPase activity to phosphoprotein level. ' $n$ ' refers to the number of subjects sampled; each sample was assayed in triplicate. The result shown is the mean $\pm$ S.E.M. Asterisk indicates significantly different from control, $p<0.01$.

| control |  |
| :---: | :---: |
| $(\mathrm{n}=7)$ | CF |
| $(\mathrm{n}=8)$ |  |

10. $\mathrm{mM} \mathrm{CaCl}_{2}$

Phosphoprotein (fmol/mg)
$6080 \pm 69$
$5110 \pm 66^{*}$
ATPase (pmol/mg/min)
$21.4 \pm .7$
$17.1 \pm .8^{*}$
Turnover (min ${ }^{-1}$ )
$3.46 \pm 12$
$3.42 \pm 11$
0.1 mM LaCl 3

| Phosphoprotein (fmol/mg) | $1498 \pm 70$ | $1114 \pm 66$ |
| :---: | :---: | :---: |
| ATPase (pmol/mg/min) | $25.7 \pm .9$ | $21.4 \pm .9$ |
| Turnover ( $\mathrm{min}^{-1}$ ) | $64.1 \pm 12$ | $48.6 \pm 11$ |
| $\underline{0.025 ~ m M ~ M g C l ~}{ }_{2}$ |  |  |
| Phosphoprotein (fmol/mg) | $209 \pm 70$ | $186 \pm 66$ |
| ATPase (pmol/mg/min) | $6.90 \pm .89$ | $7.72 \pm .84$ |

## DISCUSSION

ATP- and magnesium-stimulated active calcium extrusion is a basic plasma membrane function in most living cells and its general characteristics seem to be preserved in the human erythrocyte membrane (Schatzmann, 1975; Sarkadi, 1980). Furthermore, due to the lack of subcellular membrane elements in the erythrocyte it is possible to isolate a plasma membrane $\left(\mathrm{Ca}^{2+}+\right.$ $\mathrm{Mg}^{2+}$ )-ATPase which is free from contamination by other species of $\mathrm{Ca}^{2+}$-ATPase. These qualities make the erythrocyte an ideal system in which to study the kinetics of the plasma membrane calcium pump enzyme.

Calcium-dependent formation of a phosphorylated intermediate of the erythrocyte calcium pump from gamma- ${ }^{32}$ p-ATP has been shown by several authors (Katz and Blostein, 1973; Knauf et al, 1974; Katz and Blostein, 1975; Rega and Garrahan, 1975; Wolf et al, 1977; Schatzmann and Burgin, 1978; Szasz et al, 1978; Niggli et al. 1979) and it is now accepted that hydrolysis of ATP by the calcium pump in human erythrocytes proceeds through a series of partial reactions involving formation of a phosphorylated intermediate. Investigation of the effects of regulatory proteins and various modifications to the ionic environment on phosphoprotein formation has and will continue to contribute to our better understanding of the molecular properties of the calcium pump enzyme.

A special advantage in using membrane fragments is that changes in phosphoprotein formation can be directly compared to alterations in ATPase activity. This allows the rate of forma-
tion, steady-state level and rate of decay of the E.Ca.P complex to be compared with the net velocity of the enzyme through its entire sequence of partial reactions. From such comparisons a model of the reaction sequence has been developed, indicating the order of the various partial reactions and the sites where regulatory proteins and ions may be exerting their effect(s). The current understanding of the partial reaction sequence for this enzyme, previously described in the Introduction, is depicted in Figure 1. Briefly: Enzyme state $E_{1}$ is considered to bind $\mathrm{Ca}^{2+}$ at the cytosolic membrane surface, thereby promoting reaction with ATP, forming the intermediate complex $E_{1}$.Ca.P. The conformational transition of the "high energy" phosphoprotein $E_{1} . C a . P$ to its "lower energy" form $E_{2} . C a . P$. is believed to result in a decreased affinity of the binding site for calcium and its reorientation to face the exterior surface of the erythrocyte membrane. This conformational change appears to be stimulated by micromolar concentrations of magnesium. Translocation of calcium is thought to result from displacement of calcium from this low affinity state. Rega and Garrahan (1975) have suggested that in the $E_{2} . C a . P$ form the acyl-phosphate bond is more accessible to water and is therefore rapidly hydrolysed to $E_{2}$. Ca plus inorganic phosphate. $E_{2}$. Ca then dissociates to $E_{2}$ plus $\mathrm{Ca}^{2+}$ and $\mathrm{E}_{2}$ undergoes a conformational transition back to $E_{1}$.

In the following discussion I will attempt to explain the effects of calcium, lanthanum, calmodulin and magnesium on cal-cium-dependent phosphoprotein formation and $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase activity in terms of the various steps in the reaction sequence
of this enzyme.
I. Effects of Calcium on $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase Activity and the Intermediate Reaction Sequence

One interesting characteristic of the erythrocyte calciumtransport ATPase is that this enzyme, which requires calcium to function, is also inhibited by calcium. The inhibitory action of calcium on the activity of the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase has been reported by several authors (Vincenzi et al, 1980; Klinger et al, 1980; Graf and Penniston, 1981; Al-Jobore and Roufogalis, 1981). We found that ATPase activity was inhibited by calcium concentrations greater than $10^{-4} \mathrm{M}\left(\mathrm{K}_{\mathrm{i}}=1.7 \times 10^{-3} \mathrm{M}\right)$, which agrees with the finding of Schatzmann (1982) that calcium concentrations greater than $1-5 \times 10^{-5} \mathrm{M}$ are inhibitory.

When the effect of calcium concentration on formation of the phosphorylated intermediate (E.Ca.P) was studied a distinctly biphasic relationship $\left(K_{1}=5 \times 10^{-8} \mathrm{M} ; \mathrm{K}_{2}=7.2 \times 10^{-4} \mathrm{M}\right.$ ) was observed, in which levels of the E.Ca.P complex were maximal in the presence of $10 \mathrm{mM} \mathrm{Ca}{ }^{2+}$. Lichtner and Wolf (1980a) obtained a similar response to calcium concentration using a purified enzyme preparation. The low affinity component of E.Ca.P was of special interest as it occurred within the same range of free calcium concentrations where calcium-dependent inhibition of $\mathrm{Ca}^{2+}$-ATPase activity occurred. One possible explaination for this observation is that either the pump protein or an associated protein was being phosphorylated by a calcium-dependent protein kinase, resulting in inhibition of the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase. Autoradio-
grams of phosphorylated membranes indicate that, at all calcium concentrations tested, most of the ${ }^{32} \mathrm{p}$ was incorporated into a 135,000-145,000 dalton protein and it was rapidly released upon exposure to hydroxylamine, as previously shown by Lichtner and Wolf (1980b). The molecular weight of the phosphoprotein measured here is similar to that of 138,000 daltons reported by Carafoli et al (1982) for the erythrocyte membrane $\mathrm{Ca}^{2+}$-ATPase. The similarity in molecular weight, as well as the sensitivity of the phosphorylated enzyme to hydroxylamine are consistent with formation of an acyl-phosphate bond on the calcium pump protein rather than a kinase-mediated phosphorylation.

Calcium-dependent inhibition of ATPase activity and the low affinity component of E.Ca.P formation have been attributed to inhibition of the dephosphorylation pathway by calcium (Lichtner and Wolf, 1980a; Schatzmann, 1982). These authors have proposed that at high concentrations, calcium competes with magnesium at the 'magnesium-specific' site, thereby inhibiting the conformational change from $E_{1}$.Ca.P to $E_{2}$.Ca.P. A similar biphasic relationship between calcium concentration and E.Ca.P formation has been demonstrated for the $\mathrm{Ca}^{2+}$-ATPase in sarcoplasmic reticulum (S.R.) vesicles prepared from rabbit skeletal muscle (Almeida and de Meis, 1977). These workers found that the low affinity component resulted from a high calcium concentration on the luminal side of the S.R. membrane but not on the cytosolic side, and therefore they suggested the low affinity component was associated with the presence of a low affinity calcium binding site on the region of the enzyme exposed to the lumen (ie.
$\left.E_{2} \cdot C a . P\right)$. It should be pointed out here that in the sarcoplasmic reticulum, in contrast to the erythrocyte, the binding sites for ATP and $\mathrm{Mg}^{2+}$, as well as the high affinity calcium sites, are found on the cytosolic side of the membrane. Ikemoto (1974) has also presented evidence that the binding of $\mathrm{Ca}^{2+}$ to a lower affinity site (association constant $1.0-1.6 \times 10^{3} \mathrm{~m}^{-1}$ ) could be involved in the inhibition of the ATPase by excess $\mathrm{Ca}^{2+}$. In addition to inhibiting dephosphorylation, binding of calcium to the "low affinity" site enhanced phosphorylation of the enzyme by ${ }^{32} \mathrm{P}_{\mathrm{i}}$; this phosphate could subsequently be transfered to ADP, forming ATP under conditions which reverse the calcium pump mechanism (de Meis and Caravalho, 1976). Although much of this work has been done in the S.R. system of skeletal muscle, similar results have been obtained from erythrocytes. In resealed ghosts, in the presence of a steep inwardly oriented calcium gradient, Rossi et al (1978) observed ${ }^{32} \mathrm{P}_{\mathrm{i}}$ incorporation into ATP. Wuthrich et al (1979) showed picomolar amounts of net ATP synthesis by running the calcium pump backwards in inside-out membrane vesicles. Thus, results obtained in both erythrocyte plasma membranes and skeletal S.R. are not entirely consistent with the viewpoint of how calcium inhibits dephosphorylation by inhibiting the conformational change from $\mathrm{E}_{1}$.Ca.P to $\mathrm{E}_{2}$.Ca.P. An alternative explanation for these results involves an interaction between calcium and the $E_{2}$ state of the enzyme, possibly the $E_{2} \cdot P$ complex. Such an interaction could maintain the enzyme in a phosphorylated state ( $\mathrm{E}_{2}$.Ca.P) drive the dephosphorylation pathway in the opposite direction. In addition, these results suggest a parallel between the S.R. system and the erythrocyte
plasma membrane system in terms of the mechanism of calciumdependent inhibition of dephosphorylation.

In the presence of $10^{-6} \mathrm{M} \mathrm{Ca}^{2+}$, formation of the E.Ca.P complex approached steady-state with a $t_{1 / 2}$ greater than 15 seconds. Lichtner and Wolf (1980a), working at the same calcium concentration, found slower apparent rates of formation $\left(t_{1 / 2}=\right.$ 30-60 seconds). However, their assays were carried out at a lower temperature $\left(0^{\circ}\right.$ vs $\left.10^{\circ} \mathrm{C}\right)$. These results suggest that the rate of the first reaction:

$$
\mathrm{E}_{1}+\mathrm{Ca}^{2+}+\mathrm{ATP} \rightleftharpoons \mathrm{E}_{1} \cdot \mathrm{Ca} \cdot \mathrm{P}+\mathrm{ADP}
$$

was slow at this calcium concentration. However, under the same conditions turnover number was maximal and ATPase activity was 70-80\% of its maximum velocity ( $\mathrm{V}_{\text {max }}$ ). Taken together, these results imply the following: 1) In the presence of $10^{-6} \mathrm{M} \mathrm{Ca}$ and the assay conditions employed for the present study, formation of the $E_{1}$.Ca.P complex is the rate-limiting step in the reaction cycle either directly or by virtue of a rate-limiting $E_{2}-E_{1}$ transition; 2) as the phosphorylation reached steady-state slowly, even though turnover of the phosphorylated intermediate was high, the rate of formation of the E.Ca.P complex must have been very similar to the rate of dephosphorylation; and, 3) all partial reactions which occur between the formation of the phosphorylated intermediate and its decay, including the conformational change between $E_{1} . C a . P$ and $E_{2} . C a . P$, were at least as rapid as the formation and breakdown of the intermediate. Sarkadi (1980) has proposed that, in the presence of magnesium, the rate limiting step in the pumping cycle is either the formation of the
$\mathrm{E}_{1}$. Ca complex or the calcium-translocation step. In light of these results, the "slow" step would appear to be the calciumdependent phosphorylation step, either directly or due to a limiting amount of available free enzyme ( $E_{1}$ ).

When the calcium concentration was increased to $4.0 \times 10^{-4}$ $M$, both the steady-state level and the apparent rate of formation ( $t_{1 / 2}=5$ seconds) of the E.Ca.P complex were increased compared to that observed at $10^{-6} \mathrm{M} \mathrm{Ca}^{2+}$. At this $\mathrm{Ca}^{2+}$ concentration $\left(4.0 \times 10^{-4} \mathrm{M}\right)\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase activity was inhibited by $20-$ $30 \%$ (with respect to $V_{\text {max }}$ ) and turnover of the phosphorylated intermediate was inhibited by more than $90 \%$. These observations are consistent with results, discussed above, showing that at concentrations greater than $5 \times 10^{-5} \mathrm{M}$, calcium inhibits dephosphorylation of the phosphoprotein complex. When the dephosphorylation pathway is inhibited, the rate of formation of E.Ca.P exceeds the rate of breakdown. As a result of this, the steadystate level of E.Ca.P must rise to a point where dephosphorylation is 'driven' at a rate equal to formation of the complex, and a new equilibrium is established. There are at least two factors which may be contributing to the increase in the apparent rate of formation observed. Briefly, these are: 1) The high affinity calcium binding sites on the enzyme, which have a dissociation constant for calcium of approximately $10^{-6} \mathrm{M}$ (Schatzmann, 1973; Ferreira and Lew, 1976; Larsen et al, 1978), would not be saturated at a calcium concentration of $10^{-6} \mathrm{M}$. Increasing the calcium concentration to $4.0 \times 10^{-4} \mathrm{M}$ saturates the high affinity calcium binding sites, which would have a stimulatory effect on the apparent rate of formation. 2) As dephosphorylation becomes
inhibited by calcium and higher steady-state levels of E.Ca.P are required to drive the dephosphorylation pathway, the apparent rate of formation of the E.Ca.P complex begins to approximate the true rate of formation. One additional consequence of the inhibition of dephosphorylation is a shift in the equilibrium of the enzyme from the dephosphorylated states $\left(E_{1}\right.$ and $\left.E_{2}\right)$ towards the phosphorylated states $\left(E_{1} . C a . P\right.$ and $\left.E_{2} . C a . P\right)$ resulting in less $E_{1}$ available to bind to $C$ a and ATP, accounting for at least part of the observed reduction in steady-state activity of the enzyme.
II. Effect of Lanthanum on the Partial Reactions of the $\left(\mathrm{Ca}^{2+}+\right.$ $\mathrm{Mg}^{2+}$ )-ATPase

Lanthanum produced effects which were virtually identical to those of high ( $\left.4.0 \times 10^{-4} \mathrm{M}\right) \mathrm{Ca}^{2+}$, namely; ( $\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}$ )-ATPase activity was greatly reduced while the apparent rate of formation and steady-state level of the E.Ca.P complex were increased markedly. These effects are consistent with previous reports that lanthanum inhibits the dephosphorylation pathway of the erythrocyte $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase (Szasz et al, 1978; Schatzmann and Burgin, 1978; Muallem and Karlish, 1982). Recently Luterbacher and Schatzmann (1983) suggested that lanthanum inhibits the conformational transition required for dephosphorylation, whereas Sarkadi (1980) has proposed that lanthanum binds to the $E_{2} \cdot P$ complex following the dissociation of calcium, thereby stabilizing the enzyme in a phosphorylated form. Both of these mechanisms imply that lanthanum acts on the phosphorylated interme-
diate, however, Szasz et al (1978) have shown that lanthanum will stimulate phosphoprotein formation even in the absence of calcium. In the present study lanthanum appeared to antagonize the calcium-dependent inhibition of dephosphorylation. Together, these two results suggest a possible interaction at the highaffinity calcium binding site on the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase, although they do not rule out other possible sites of action for lanthanum.

## III Effect of Calmodulin on the Intermediate Reactions of the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase

Previously, it was thought that the number of calmodulin binding sites exceeded the number of pump sites by about five fold (see Al-Jobore et al, 1984). These results were based on 1) direct calmodulin binding studies (Graf et al, 1980) and kinetic titration of $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase activation (Jarret and Kyte, 1979), which showed that there were about 4000 - 6000 calmodulin binding sites per erythrocyte, and 2) phosphorylation studies (Knauf et al, 1974; Rega and Garrhan, 1975) which suggested there were approximately 700 calcium pumping sites per erythrocyte. It is now known, however, that the levels of phosphoprotein used to determine this value were not maximum values and therefore do not provide an accurate means of determining the total number of pump sites per cell. From results obtained in the present study, assuming $10^{-9} g$ of membrane protein per erythrocyte ghost (Lichtner and Wolf, 1979), we have estimated that there are roughly 2400-3600 pump sites per cell. This value agrees well
with that of Schatzmann (1982), who estimates at least 2700 sites per erythrocyte. Thus, the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase does, in fact, appear to represent a major fraction of the total number of calmodulin binding sites within the erythrocyte.

The effects of calmodulin on the kinetics of the $\mathrm{Ca}^{2+}+$ $\mathrm{Mg}^{2+}$ )-ATPase, namely an increase in the maximum rate of hydrolysis of ATP and an increase in the apparent calcium affinity, have been well documented (for reviews see Roufogalis, 1979; Sarkadi, 1980; Al-Jabore et al, 1984; Schatzmann, 1982; Penniston, 1983; Schatzmann, 1985). There is now convincing evidence that both formation (Muallem and Karlish, 1980) and breakdown (Jeffery et al, 1981; Rega and Garrahan, 1980) of the calcium-dependent phosphorylated intermediate are accelerated by calmodulin, enhancing the turnover of the enzyme. Klinger et al (1980) and Schatzmann (1982) have presented evidence suggesting that calmodulin does not influence calcium-dependent inhibition of ATPase activity. Similarly, we have found that calmodulin does not alter the apparent affinity of calcium-dependent inhibition of $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase activity or the biphasic relationship between calcium concentration and E.Ca.P formation. These studies allow identification of which particular sequences of steps within the reaction cycle are accelerated by calmodulin, but they do not define the particular reaction steps affected by calmodulin. They do , however, suggest that the step at which calmodulin acts to stimulate dephosphorylation is not the same step with which calcium interferes to inhibit dephosphorylation. In a study by Muallem and Karlish (1980), calmodulin was shown to increase the apparent rate of formation of the E.Ca.P
complex without affecting the steady-state level. In the present study, however, the effect of calmodulin on the kinetics of E.Ca.P formation differed greatly depending on the calcium concentration in the assay medium. When the calcium concentration was maintained at $10^{-6} \mathrm{M}$, calmodulin increased the apparent rate of formation of the E.Ca.P complex, however steady-state was not reached within the range of reaction times examined (1-30 seconds). At a higher $\mathrm{Ca}^{2+}$ concentration ( $\left.4.0 \times 10^{-4} \mathrm{M}\right)$ calmodulin had no effect on the apparent rate of formation, but it reduced the steady-state level of E.Ca.P by more than $25 \%$. The reduction by calmodulin of the steady-state level has been reported previously by other investigators (Jeffery et al, 1981; Rega and Garrahan, 1980 ) at lower calcium concentrations (5-6 $\times 10^{-5} \mathrm{M}$ ). The increase in apparent rate of phosphoprotein formation observed in the presence of $10^{-6} \mathrm{M} \mathrm{Ca}^{2+}$ upon addition of calmodulin could be achieved in at least three ways. These are: 1) an increase in the calcium affinity of the high-affinity calcium binding sites, such that a higher degree of saturation is achieved at this low calcium concentration, which could, in theory, "drive" the rate of formation to a higher value (assuming that the $E_{1}-E_{2}$ transition is not rate-limiting), 2) an alteration in the equilibrium between the $E_{1}$ and $E_{2}$ forms of the enzyme towards the $\mathrm{E}_{1}$ form, resulting in more high affinity sites available to interact with $\mathrm{Ca}^{2+}$ and ATP (Muallem and Karlish, 1980), or 3) an alteration in the equilibrium between $\mathrm{E}_{1}+\mathrm{Ca}^{2+}+\mathrm{ATP}$ and $E_{1} . C a . P$ in favor of the intermediate complex. At a higher calcium concentration $\left(4.0 \times 10^{-4} \mathrm{M}\right)$ calmodulin did not alter the
apparent rate of formation of the E.Ca.P complex, which supports the first mechanism, that the increased rateof E.Ca.P formation is due to an increase in affinity of the calcium binding sites and once the calcium concentration is high enough to saturate these sites, no further effect is seen. In addition, the observation that calmodulin lowered the steady-state amount of E.Ca.P, as others have also shown (Jeffery et al, 1981; Rega and Garrahan, 1980), indicates that the rate of dephosphorylation was increased. Jeffery et al (1981) found that both magnesium 10.25 mM ) and calmodulin stimulated the rate of dephosphorylation, however in the presence of high magnesium, calmodulin did not produce an additional increase in dephosphorylation, and vice versa. From these results the authors concluded that calmodulin and magnesium may be stimulating the rate of dephosphorylation by acting on the same kinetic step, namely the conformational transformation from the form of the enzyme with a low reactivity to water ( $\left.E_{1} . C a . P\right)$ to that conformation characterized by a high reactivity to water $\left(E_{2} . C a . P\right)$. In the presence of lanthanum 10.1 mM ), which inhibits dephosphorylation and at a relatively high free calcium concentration of $1-2 \times 10^{-4} \mathrm{M}$, calmodulin increased the apparent rate of formation and the steady-state level of E.Ca.P. This result indicates that the true rate of formation of the E.Ca.P complex is, in fact, increased by calmodulin even at calcium concentrations in excess of those sufficient to saturate the high affinity binding sites. Therefore, this rate increase is not simply due to an increase in calcium affinity.

An alternate mechanism was suggested by Muallem and Karlish (1980), who considered that since the rate of transformation from
$\mathrm{E}_{2}$ to $\mathrm{E}_{1}$ is slow, by accelerating this step with calmodulin both the rate of formation of E.Ca.P and the apparent calcium affinity of the enzyme are increased. Although one can easily see how such an action would increase the rate of phosphorylation, it is difficult to see how it will account for the change in calcium affinity. The conformational change from $E_{2}$ to $E_{1}$ involves a reorientation of the calcium binding site to the cytosolic side of the membrane and an increase in calcium affinity by several orders of magnitude. As the high and low affinity sites differ by several orders of magnitude, it is unlikely that both contribute to the apparent affinity for calcium of the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase activity, as may happen with two or more sites having similar affinities. Therefore, according to Muallem and Karlish's theory, the increased affinity is due to an increase in the number of sites with which calcium can interact. Such an effect would uniformly increase the rate of ATP hydrolysis but should not alter its calcium dependence. Schatzmann (1982) has questioned the notion that calmodulin increases the maximum velocity $\left(V_{\max }\right)$, noting that in calmodulin-depleted enzyme the calcium activation curve moves towards the region of inhibitory calcium concentrations, as the affinity of calcium-dependent inhibition is not affected by calmodulin. This, then, would be responsible for the lower values of $V_{\max }$ estimated in the absence of calmodulin. Such ideas suggest that calmodulin produces a single change in the enzyme which manifests itself as an alteration in both calcium affinity and $V_{\text {max }}$. Contrary to this idea, Minocherhomjee et al (1982) showed that polyanions could mimic
the effect of calmodulin on calcium affinity but not on $V_{\text {max }}$, and as in the case of calmodulin, this activation could be antagonized by trifluoperazine. Thus calmodulin may produce more than one effect upon the enzyme, allowing more rapid conformational changes in both the phosphorylated states and the non-phosphorylated states, and also increasing the affinity of the high affinity calcium binding sites.

IV Effect of Magnesium on the Intermediate Reactions of the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase

In addition to other activators, the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase requires micromolar amounts of magnesium in order to function at its maximum rate. Several authors (Rega and Garrahan, 1975; Schatzmann and Burgin, 1978; Szasz et al, 1978) have demonstrated that formation of the E.Ca.P complex does not require the presence of magnesium, however in a recent review article Schatzmann (1982) suggested that $\mathrm{Mg}^{2+}$ is probably required at the phosphorylation step. As the previous studies did not employ a magnesium-specific chelator in the assay systems used, there is a possibility that endogenous levels of $\mathrm{Mg}^{2+}$ were high enough to meet any requirements for phosphorylation. In the present study, membranes were washed in a magnesium-free buffer and assayed in the presence of the magnesium chelator, CDTA, to bind any residual magnesium. In their "magnesium-free" condition (no added magnesium), Richards et al (1978) found that the $V_{\text {max }}$ for ATP hydrolysis was depressed, whereas with CDTA in the medium both the apparent calcium affinity and the $\mathrm{V}_{\text {max }}$ were reduced. There-
fore small amounts of magnesium do maintain the enzyme in the high-affinity $E_{1}$ form. In addition, the calcium activation of ATPase activity appears to be biphasic, whereas it is hyperbolic in the presence of magnesium ( 0.025 mM ) . The difference between our findings and those of Richards et al may be attributable to contamination by residual free magnesium in their assay, or perhaps to some effect of CDTA upon the enzyme itself in our study. When the formation of the E.Ca.P complex in the absence of magnesium was compared to that with $25 \mu \mathrm{M}$ magnesium, we found; 1) no change in the calcium-dependency of E.Ca.P formation at calcium concentrations within the range of $5.0 \times 10^{-8}$ to 1.0 x $10^{-2} \mathrm{M}$ (free), and 2) no significant change in the rate of formation of the E.Ca.P complex at either low ( $10^{-6} \mathrm{~m}$ ) or high $\left(4.0 \times 10^{-4} \mathrm{M}\right)$ calcium concentrations. Hence these results confirm those of previous investigators, demonstrating that magnesium is not required for the formation of the E.Ca.P complex. On the other hand, Larocca et al (1981) found that magnesium (0.5 mM ) increased the rate of formation of the phosphorylated intermediate, while steady-state levels were independent of magnesium concentration. In contrast to this, Jeffery et al (1981) found that in addition to enhancing dephosphorylation of E.Ca.P, magnesium ( 0.25 mM ) decreased the steady-state levels. These discrepancies in results concerning the effects of magnesium on steadystate levels of the E.Ca.P complex are difficult to rationalize and may in fact be highly dependent on other factors, such as temperature or ATP concentration, which are known to have a profound effect on the activity of the enzyme. Although these
other reports appear contradictory in nature, stating that eiher phosphorylation or dephosphorylation was affected, they all support the results of the present study which suggest that magnesium stimulates the turnover of the enzyme by simultaneously increasing both the rate of formation and the rate of breakdown of the phosphorylated intermediate. Hence no change is detected in either the apparent rate of formation or in the calciumdependency of phosphoprotein formation, but a large effect is seen in the velocity of ATP hydrolysis. Rega and Garrahan (1975) showed that magnesium stimulated a transition of the intermediate complex E.Ca.P from the $E_{1}$ state of the enzyme to the $E_{2}$ conformation, which then rapidly dephosphorylates. It is possible that magnesium may also stimulate the reverse of this change, namely the conversion from $E_{2}$ to $E_{1}$. As with calmodulin, magnesium appears to in some way facilitate a conformational change in the enzyme in both the phosphorylated and the non-phosphorylated states. The actual mechanism of this action is not known.

As described previously, the inhibition of dephosphorylation by calcium has been attributed to calcium blocking the conformational change from $E_{1}$.Ca.P to $E_{2} \cdot C a . P$ by competing at a magnesium-specific binding site (Schatzmann, 1982). This theory implies two things, namely; 1) there is a magnesium-specific site involved, and 2) the buildup in E.Ca.P observed in the presence of high concentrations of calcium represents the $\mathrm{E}_{1}$. Ca. P form of the enzyme. Considering the very low turnover number observed in the presence of millimolar concentrations of calcium (1-5 min ${ }^{-1}$ ), conversion to $E_{2} \cdot C a . P$ and or subsequent hydrolysis proceed very slowly. In regards to the magnesium-specificity of the site,
varying the magnesium concentration from zero to $1.0 \times 10^{-3} \mathrm{M}$ had no effect on E.Ca.P formation in the presence of 10 mM calcium. Other investigators have demonstrated that magnesium will compete with calcium to antagonize the buildup of E.Ca.P (Lichtner and Wolf, 1980a) and the inhibition of ( $\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}$ )-ATPase activity by calcium (Klinger et al, 1980). These studies, however, utilized equimolar or greater amounts of magnesium to "compete" with calcium. This is not consistent with the concept of a mag-nesium-specific site. Either the site at which the effect takes place does not discriminate between magnesium and calcium, or what is seen represents magnesium antagonizing the binding of calcium at a low-affinity calcium binding site. To determine whether calcium blocks the equilibrium between $E_{1}$.Ca.P and $E_{2}$.Ca.P or stabilizes $E_{2} \cdot C a . P$, the nucleotide sensitivity of the phosphoprotein formed at high calcium concentrations was examined. Dephosphorylation of the phosphorylated intermediate was initiated using either ATP (1 mM) or ADP (1 mM). ATP is known to stimulate dephosphorylation by driving the reaction in the forward direction whereas, ADP is believed to stimulate dephosphorylation in both the forward direction and by reversing the phosphorylation by reacting with the high energy phosphate in $E_{1}$. Ca. $P$ (Rega and Garrahan, 1978; Schatzmann, 1982). In the presence of $10 \mathrm{mM} \mathrm{Ca}{ }^{2+}$, increasing the free magnesium concentration from zero (0.1 mM CDTA) up to 1 mM had no effect upon the sensitivity of the phosphorylated intermediate to dephosphorylation by either ADP or ATP. Garrahan and Rega (1978) previously demonstrated that in the presence of high concentrations of ATP ( 1.0 mM ), magnesium
stimulated dephosphorylation by promoting the conformational change from $E_{1}$. Ca. P to $E_{2} . C a . P$. Theoretically, if this conformational change was blocked by calcium, magnesium should have enhanced the sensitivity of the phosphorylated intermediate to dephosphorylation by ATP in the foreward direction. Although dephosphorylation was greater in the presence of ADP after a five second chase than for ATP, after fifteen seconds, dephosphorylation by ATP was 80-90\% complete and equal to that of ADP. From these results, inhibition of the conformational change from $E_{1} \cdot C a . P$ to $E_{2} \cdot C a . P$ by high calcium concentrations does not appear to be sufficient to account for the accumulation (low turnover) of the phosphorylated intermediate observed in the presence of high calcium concentrations.

V Changes in the $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase Activity and Phosphoprotein Formation in Erythrocytes from Patients with Cystic Fibrosis

The methodology developed to estimate maximum levels of phosphorylated enzyme intermediate, which is a measure of the available functional enzyme activity, was used to examine the functional level of the calcium pump in a disease state thought to involve a defect in calcium handling by the cell. Previous studies have indicated that there is a decrease in $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)-$ ATPase activity in erythrocyte membranes of patients with cystic fibrosis (CF) (Horton et al, 1970; Katz, 1978; Ansah and Katz, 1980; Foder et al, 1980; Gietzen et al, 1980; for review see Katz et al, 1984). This decrease in ATPase activity has been cor-
related with a decrease in calcium transport activity in insideout vesicle preparations of erythrocyte membranes (Ansah and Katz, 1980). Recently, Miner et al (1983) presented a report indicating that in the presence of lanthanum, which blocks dephosphorylation, significantly less phosphoprotein was formed in CF erythrocytes. We found that in the presence of lanthanum (0.1 mM) or high calcium concentrations (10 mM), compared to age and sex-matched controls, there was a significant reduction in the phosphorylated intermediate formed in erythrocytes from CF patients, and this reduction was correlated with a similar reduction in ATPase activity. The turnover number of the intermediate, however, did not differ significantly from controls. There was no difference in the "basal" levels of ATPase activity or phosphoprotein formation assayed in the presence of magnesium (2.5 $\times 10^{-5} \mathrm{M}$ ) alone. These results suggest that the number of active calcium pumping sites may be lower in CF membranes due to either 1) the presence of non-functional Ca-ATPase molecules, or 2) fewer Ca-ATPase molecules in the CF erythrocyte membranes. The possibility of decreased enzyme activity due to impaired function of the enzyme seems doubtful, as the turnover number of the existing enzyme was not altered.

1. At low calcium concentrations (1.0 $\mu \mathrm{M}$ ), calmodulin stimulated the apparent rate of formation of the phosphorylated intermediate, whereas at higher concentrations of calcium (0.4 $\mathrm{mM})$ calmodulin did not alter the apparent rate of formation but decreased the steady-state levels. These results are consistent with previous studies which found that calmodulin stimulated both formation and breakdown of the intermediate complex. These results also suggest that at the lower calcium concentrations phosphorylation was the rate-limiting step, whereas at higher calcium concentrations dephosphorylation became rate-limiting. The steps stimulated by calmodulin likely represent those which are constrained by the "inhibitory subunit". Although there is still no direct evidence as to the step at which calmodulin acts to produce the observed increase in the rate of phosphoprotein formation, current knowlege concerning the effects of calmodulin may be best explained by suggesting that camodulin produces a change in the enzyme which has more than one effect on its reaction cycle.
2. At concentrations greater than $5.0 \times 10^{-5} \mathrm{M}$, calcium appeared to block dephosphorylation of the intermediate complex. Previous reports stated that this inhibitory effect of calcium could be antagonized by magnesium and was likely due to calcium antagonizing the magnesium-stimulated transition from $E_{1}$. Ca. $P$ to $E_{2}$.Ca.P (Schatzmann, 1982). Results from the present study were not consistent with this idea: 1) phosphoprotein formed due to
calcium-dependent inhibition of dephosphorylation could be dephosphorylated in either the foreward or backward direction; 2) the absence of magnesium in the reaction medium did not simulate the effects of millimolar concentratons of calcium; and 3) magnesium did not appear to antagonize the inhibition of dephosphorylation by calcium. An alternate mechanism was proposed where calcium interacts with the $E_{2} \cdot P$ complex to produce $E_{2} \cdot C a . P$, thereby maintaining the enzyme in the phosphorylated state. This proposal assumes that calcium dissociation precedes the actual dephosphorylation step.
3. The presence of magnesium does not appear to be an absolute requirement for phosphorylation. However, since magnesium does increase the apparent calcium sensitivity of the $\left(\mathrm{Ca}^{2+}\right.$ $+M g^{2+}$ )-ATPase in addition to increasing the turnover of the phosphorylated intermediate, it is possible that magnesium stimulates the rate of transformation from $E_{2}$ to $E_{1}$, as suggested by Sarkadi (1980) in addition to its stimulatory effects on the transition from $E_{1}$.Ca.P to $E_{2}$.Ca.P.
4. Lanthanum has been shown to inhibit the $\left(\mathrm{Ca}^{2+}+\right.$ $\mathrm{Mg}^{2+}$ )-ATPase in intact red cells when present in the extracellular medium. Sarkadi (1980) proposed that lanthanum binds to the $E_{2} . P$ complex, once calcium has dissociated, stabilizing the enzyme in the phosphorylated state. Results from the present study, as well as others suggest that lanthanum may stimulate phosphorylation of the enzyme in the absence of calcium. These results suggest that in addition to its effects on $E_{2} \cdot P$, lantha-
num may substitute for calcium in promoting phosphorylation. The phosphorylated intermediate formed in the presence of lanthanum is rapidly dephosphorylated by ADP but decomposes slowly in the foreward direction (Shatzmann and Burgin, 1978), suggesting that the phosphoprotein formed in the presence of lanthanum converts to the $E_{2}$ state very slowly.
5. Reduced levels of both $\left(\mathrm{Ca}^{2+}+\mathrm{Mg}^{2+}\right)$-ATPase activity and of the phosphorylated intermediate were observed in membrane fragments from patients with cystic fibrosis with respect to normal subjects. These findings suggest one of two possibilities: 1) there are fewer calcium pumping sites present in the erythrocyte membranes from individuals with cystic fibrosis; or 2) a population of inactive calcium pumping sites exists in these erythrocytes.

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