

Energized Endocytosis in Human Erythrocyte Ghosts

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ABSTRACT The mechanism of endocytosis in resealed human erythrocyte ghosts was studied. The energy for endocytosis or micropinocytosis appears to be derived from Mg-ATP, and membrane internalization is preceded by activation of a membrane-associated Ca, Mg-ATPase and by the active efflux of Ca. Endocytosis, Ca, Mg-ATPase activity, and active Ca efflux all require the presence of Mg. Furthermore, these three phenomena, endocytosis, Ca, Mg-ATPase activity, and active Ca extrusion, all have a concentration dependence on Ca such that low concentrations stimulate and higher concentrations inhibit the phenomena. The optimal concentration of Ca is identical for endocytosis, active Ca efflux, and Ca, Mg-ATPase. Morphologic studies indicated that while active Ca efflux and activation of the Ca, Mg-ATPase activity occurred promptly upon onset of incubation, there was a significant time delay before endocytosis occurred, which suggests that endocytosis additionally involved a more slowly functioning mechanochemical mechanism. Ruthenium red, a specific inhibitor of Ca, Mg-ATPase and Ca transport, inhibited endocytosis in a concentration-related manner. Prostaglandins E₁ and E₂ had no measurable effect on ghost endocytosis, active Ca efflux, or Ca, Mg-ATPase activity.

INTRODUCTION

The phenomenon of endocytosis or membrane internalization occurs in a variety of cell types and may be related to phagocytosis or to mechanisms wherein macromolecules or particulates may enter the cell through its otherwise impenetrable plasma membrane (1, 2). Human erythrocytes are not usually thought of in the context of phagocytosis or pinocytosis; however, in certain circumstances endocytosis can be seen in circulating human erythrocytes (3-5). A variety of agents (6) can induce membrane internalization *in vitro* in in-

Received for publication 10 May 1974 and in revised form 12 March 1975.

tact human erythrocytes and the extent of endocytosis correlates directly with cellular youth and with the energy metabolism of the red cell, as represented particularly by its ATP content (7).

Endocytosis in human erythrocyte ghosts, as distinguished from intact erythrocytes, was studied by Peniston and Green as a model system for studying energization of plasma membranes by ATP (8). Subsequent studies have indicated that the specific substrate for endocytosis in resealed human erythrocyte ghosts is Mg-ATP (7), and that ADP can substitute partially but other nucleotide di- and triphosphates as well as cAMP function poorly in this regard (9). In addition to a strict requirement for Mg, it was observed that low concentrations of Ca stimulated erythrocyte (9) ghost endocytosis, whereas higher concentrations of Ca inhibited endocytosis. This biphasic response to calcium occurred within a concentration range of 0.1-2.0 mM. Other forms of endocytosis in erythrocyte ghosts have been described, some of which appear to depend on the ionic strength of the medium and not on any sort of bioenergetic considerations (10). This membrane endovesiculation without requirement of ATP may be related to or entirely distinct from energized endocytosis.

Because energized endocytosis in human erythrocyte ghosts as studied by at least two laboratories (8, 9) requires the specific interaction of Mg and ATP, we studied the interaction of Ca, Mg, and ATP in erythrocyte ghost endocytosis as a means of investigating a general phenomenon that appears to encompass an energy-linked sequence of events involving plasma membrane invagination followed by closure and resealing of the vacuole mouth, thereby leading to pinocytosis with membrane internalization.

Experiments were designed to compare the concentrations of Ca, ATP, and Mg that produced optimal activation and then inhibition of the membrane-associated Ca, Mg-ATPase (11, 12) and the related Ca efflux pump (12-15) with the concentrations of the reactants that

produced activation and inhibition of ghost endocytosis (9). Attempts were made to study the time relationship between activation of the Ca, Mg-ATPase and Ca efflux from ghosts with the onset of morphologically identifiable ghost endocytosis. We also studied the effect of a known inhibitor of membrane-associated Ca,Mg-ATPase (16) on the phenomenon of ghost endocytosis.

METHODS

Materials. Materials were obtained from sources previously described (7). [32 P]ATP labeled in the γ position ($[\gamma\text{-}^{32}\text{P}]\text{ATP}$) was made as previously described (17). The specific activity achieved was approximately 13–34 $\mu\text{Ci}/\mu\text{mol}$. [^{45}Ca]CaCl₂ was purchased from New England Nuclear (Boston, Mass.) and had a specific activity of 17.5 mCi/mg. Practical grade ruthenium red was obtained from Sigma Chemical Co. (St. Louis, Mo.) and purified, assayed, and standardized according to the method of Luft (18). Prostaglandins E₁ and E₂ were provided by Dr. P. Kury and Dr. Harden McConnell, Department of Chemistry, Stanford University.

Resealed erythrocyte ghosts. Fresh heparinized blood from normal human donors was used. The erythrocytes were washed three times with a solution consisting of 154 mM NaCl, with 2 mg glucose/ml, buffered to pH 7.4 with 5 mM imidazole-glycylglycine. The procedure used in making resealed ghosts was basically that previously described (7, 9); however, no NaF was used in these experiments to avoid interference with energy-dependent processes. Since the events we were following occurred rapidly, equilibration and resealing periods for preparing resealed ghosts were necessarily shorter than those previously used. Hemolysis was carried out at 22°C and resealing at 37°C. Since our primary

EVALUATION OF ENDOCYTOSIS, ERYTHROCYTE SIZE ATPase ACTIVITY AND Ca²⁺ CONTENT IN RESEALED GHOSTS

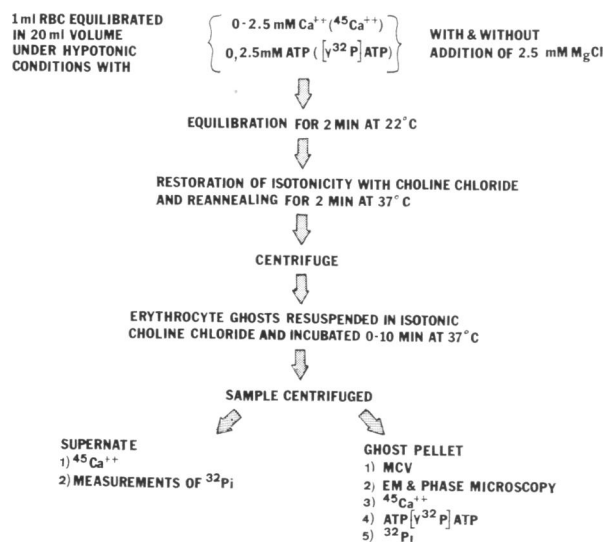


FIGURE 1 The flow chart indicates the procedures followed in hemolyzing and resealing erythrocyte ghosts, incubating the ghosts, and then analyzing the ghost pellet and suspending medium.

TABLE I
Conditions for Resealing Erythrocyte Ghosts when 2.5 mM ATP Is Present in the Hemolysis Solution

Addition to hemolysis solution		Content of addition in resealed ghosts	ATP leak*
Ca	Mg		
mM	mM	$\mu\text{mol}/\text{ml}$ packed ghosts	%
A. Resealing of NaCl tested by addition of 2.0 mM [^{22}Na]NaCl to the hemolysis solution			
0	2.5	0.78	
0.1	2.5	1.35	
0.2	2.5	1.85	
B. Resealing of ATP tested by addition of 2.5 mM [^{32}P]ATP to the hemolysis solution			
0	0.5	0.83	15
0	2.5	1.23	11
0	0	0.83, 0.87	11, 18
0.05	0	0.83	12
0.10	0	1.13	3
0.75	0	1.91	0
C. Resealing of Ca tested by addition of ^{45}Ca in the concentrations indicated to the hemolysis solution			
0.25	2.5	0.005	0.08
0.50	2.5	0.105	0.30
0.75	2.5	0.38	0.57, 0.49
0.75	0	0.53	0.58, 0.66
1.0	2.5	0.61	0.94, 0.90
5.0	2.5	2.90	5.2

* ATP leak to supernatant medium during the 10-min incubation at 37°C, expressed as a percentage of the ATP present in ghosts at the beginning of the incubation.

interest was in ghost endocytosis, an ATP-requiring phenomenon in our experiments (9), ATP was always added to the hemolysis solution at a concentration of 2.5 mM. Usually Mg was added at a concentration of 2.5 mM, except where its concentration requirement was under study. Fig. 1 indicates the methods used for hemolyzing, resealing, incubating, and analyzing the preparation. It should be emphasized that in working with the three reactants Ca, Mg, and ATP, there is a special difficulty (12), because when a given concentration of one of these three interacting materials is chosen to explore a specific phenomenon, the optimal concentration of the other two is altered in a complex manner. For example if the concentration of Ca were altered, there would be a family of curves describing the optimal concentrations for Mg and ATP for each new Ca concentration. Since it appeared that endocytosis in resealed ghosts (9) was optimal when 1.0 mM Ca, 2.5 mM Mg, and 2.5 mM ATP were added to the hemolyzing solution, these concentrations served as the basis for concentration variations of Mg and Ca. In a prior study we had observed that optimal resealing of NAD⁺ and ADP within ghosts occurred when 0.5 mM Ca and 2.0 mM Mg were added during hemolysis reversal (19) at 4°C. Subsequently, Bodemann and Passow (20) defined the conditions for optimal ghost resealing and they routinely used 4 mM Mg. Complexing agents like ATP had a deleterious effect on resealing (20) at room temperature and

37°C and the authors speculated that such agents removed the divalent cations, Ca and Mg, which participated in the maintenance of membrane impermeability. Since Mg addition was used to initiate endocytosis, it was necessary to add the other divalent cation, Ca, to the hemolyzing solution to produce ghosts that would remain relatively impermeable to Ca and ATP during the course of the experiment. The effectiveness of Ca and Mg in producing sealed ghosts in the presence of ATP is shown in Table I, where the ghosts were resealed as indicated in Fig. 1, in the presence of 2.5 mM ATP and varying concentrations of Ca and Mg. The effectiveness of resealing was tested with three dissimilar materials: NaCl, Ca, and ATP. Increasing the concentration of Ca in the hemolyzing solution increased the effectiveness of resealing of Na, ATP and Ca within ghosts. (Table I.) The very high effectiveness of ⁴⁵Ca resealing suggested that perhaps some Ca had been bound to the ghost interior or its contents. The leak of [³²P]ATP from resealed ghosts was less than 2% over a 10-min period at 37°C when optimal concentrations of Ca were used. The ability of Ca to improve the resealing of erythrocyte ghosts has been previously observed (21, 22).

After the resealing step, the suspension was centrifuged at 15,000 *g* at 4°C for a total time of 3.5 min; the supernate was discarded. Then 15 vol of isotonic choline chloride, cooled to 4°C and buffered to pH 7.4 with 5 mM imidazole-glycylglycine buffer, was added, and the sample was mixed and centrifuged as before. The supernate was again discarded and the sedimented ghosts were brought to volume with buffered isotonic choline chloride for the incubation (Fig. 1). The time elapsed from the end of the resealing step to the beginning of the incubation was 8 min at temperature of 4°C. 1 ml of packed ghosts contained approximately 10¹⁰ ghosts.

No attempt was made to classify the ghosts according to their degree of "leakiness" (20), but since almost all experiments were designed so that [³²P]ATP was resealed within ghosts, the extent of ATP leak could be conveniently followed by measuring the appearance of [³²P]ATP in the supernatant media during the course of the incubation. In more than 50 experiments the leak of [³²P]ATP during the course of the incubation was always less than 2% of the total [³²P]ATP resealed within ghosts as determined at the start of the incubation.

If Mg was omitted from the hemolyzing solution, endocytosis, as measured by the radioisotopic method (7) or phase and electron microscopy (7, 9), did not occur. Therefore, the addition of Mg was used to activate endocytosis.

Measurement of ATPase. To measure ATPase activity within erythrocyte ghosts, the resealed ATP was labeled with [³²P]ATP. The degradation of ATP as measured by production of inorganic phosphate (³²Pi) and the disappearance of [³²P]ATP, both measured by the isobutanol-benzene method, provided a double method for assaying ATPase (17). ATPase activity was expressed as micromoles or nanomoles of ³²Pi generated or [³²P]ATP consumed per minute per 10¹⁰ ghosts at 37°C. This form of expression of activity was used rather than the more usual expression of micromoles ATP per minute per milligram of membrane protein (17). The latter expression is accurate with "white" ghosts, where the amount of membrane protein is generally constant at approximately 7 mg/ml of packed erythrocyte ghosts. However, in the current study red ghosts were used, and minor variations in conditions produced enough changes in hemoglobin content of membranes so that the number of ghosts was more useful as a denominator than membrane protein content. All experi-

ments were performed with two samples of erythrocyte ghosts, one being resealed with Ca and ATP, and the other containing Mg in addition to identical concentrations of Ca and ATP. The difference in ATP degradation between the two samples was taken to represent Ca, Mg-ATPase activity. Since it was not possible for us to reseat erythrocyte ghosts in the presence of EDTA or EGTA because of the requirements for Ca and Mg in resealing the ghosts used in this study, it was not possible to rid the ghost suspension of either Mg or Ca (23). Therefore ghosts resealed in the absence of added Mg have residual Mg and a small but measurable amount of both Ca, Mg-ATPase and Mg-ATPase activity. Thus the use of the difference in ATP degradation occurring in ghosts prepared with and without Mg addition probably underestimates Ca, Mg-ATPase. For similar reasons, Ca efflux is also probably underestimated.

To minimize activation of the Na, K-ATPase, the ghosts were resealed with isotonic choline chloride and all subsequent incubations were carried out at 37°C in isotonic choline chloride buffered to pH 7.4 with 5 mM imidazole-glycylglycine. The measured Na concentration of the resealing solution was 4.0 mM, while the K concentration was 2.85 mM. The ghost wash solution as well as the suspending medium during the incubation phase had a Na content of 1.0 mM or less, and a K content 0.2 mM or less. After resuspension in the incubating medium, the Na content within ghosts was 3.0 μmol/ml packed ghosts while the K content was 0.9 μmol/ml packed ghosts.

Because the Ca, Mg-ATPase reaction begins as soon as hemolysis starts, thereby exposing the membrane-associated enzyme to the reactants, it was necessary to correct for the [³²P]ATP degradation and ³²Pi generation that had occurred during the 12 min of hemolysis, resealing, and washing of ghosts. Accordingly in all experiments a "zero time" sample was prepared so that it contained the same amounts of reactants and ghosts as the other flasks but was not incubated at 37°C, and instead was immediately subjected to centrifugation and separation and analysis (Fig. 1). In assaying optimal Ca, Mg-ATPase activity, samples were taken after 0, 3, 7, and 10 min of incubation at 37°C and the activity was linear up to 10 min if the later values were corrected for the zero value. In recording Ca, Mg-ATPase activity in this study, the values obtained over the first 3-5 min of incubation were used.

Calcium flux measurements. For most of these experiments, radioisotopic ⁴⁵Ca was used. The small amount of residual hemoglobin in the resealed ghosts did not cause quenching when the samples were dissolved in Aquasol and the radioisotopic activity was determined by liquid scintillation spectrometry. Sufficient ⁴⁵Ca was added to adjust the specific activity of Ca in the hemolyzing solution to approximately 10⁶ cpm/μmol. Since these experiments were performed with ghosts prepared by hemolyzing erythrocytes by the addition of 20 vol of hypotonic solutions, the residual hemoglobin was 5-10% of the initial value. In performing flux studies with intact erythrocytes, one generally corrects for cell water, which accounts for approximately 68% of the erythrocytes' volume, but in the case of these resealed ghosts, where approximately 90-95% of the volume is cell water, no correction for cell water content of ghosts was made. As noted above under ATPase measurements, in each experiment a zero sample was used as the basis upon which to calculate Ca efflux. Ca efflux, depending on the Ca concentration, was determined to be linear for the first 5-7 min of incubation. Thus, for calculation of optimal Ca efflux, the efflux occurring over the first 3 min of incubation was used, unless otherwise stated. To avoid problems

that arise with the exclusive use of isotopes for flux measurements, in three experiments Ca was determined by atomic absorption so that there could be accurate evaluation of total calcium present in a sample at a given time. Duplicates were not done but the three experiments noted were comparable in final results. Samples for atomic absorption were ashed and incinerated in a muffle furnace, re-suspended in a solution of lanthanum chloride, and then assayed in the Perkin-Elmer Model 403 Atomic Absorption Spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn.) (24). Ceramic or glass crucibles have Ca that contaminates the sample during the ashing procedure; however, we found it possible to use a single batch of 30-ml Vycor crucibles, using each crucible once and then discarding it (Corning Glass Works, Corning, N. Y.).

Analytic procedures. 4-ml samples containing approximately 0.3 ml of packed ghosts were incubated at 37°C, after which the ghosts were separated from the supernatant

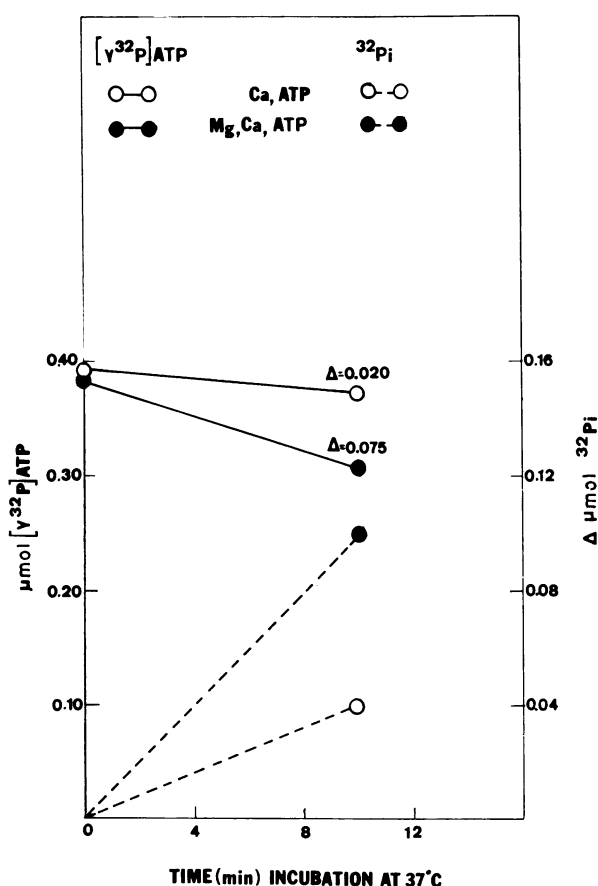


FIGURE 2 Ca, Mg-ATPase activity is related to time of incubation at 37°C. The left ordinate indicates the micromoles of isotopically labeled ATP residual in resealed ghosts at the time indicated (solid lines). Since there were 0.3 ml packed ghosts in the sample, the amount of ATP present within 1 ml of packed ghosts can be obtained by multiplying by 3.33. The change in [³²P]ATP content is indicated by Δ. The right ordinate indicates micromoles of ³²Pi produced (dotted lines). Ghosts hemolyzed with Ca and ATP are indicated by open circles and ghosts hemolyzed with Ca, ATP, and Mg are indicated by closed circles.

TABLE II
ATPase Activity of Resealed Ghosts as a Function of the Concentration of Mg Added to the Hemolyzing Solution*

Mg content of hemolyzing solution	ATPase
<i>mM</i>	<i>nmol</i> ³² Pi/min/10 ¹⁰ ghosts
0	10
0.25	12
0.50	17
1.00	23
2.50	52
5.00	42

* The hemolyzing solution also consisted of 2.5 mM ATP and 1.0 mM Ca. The effectiveness of resealing was such that the ATP content was 1.79 μmol of ATP/ml packed ghosts.

solution by centrifugation at 4°C. The supernatant solution was analyzed for ⁴⁵Ca and ³²Pi as required. (Fig. 1). The ghost pellet was brought to a defined volume, and the number of ghosts present was determined in the Coulter Counter, Model B (Coulter Electronics Inc., Hialeah, Fla.). The time lag between removal of sample from incubation to a point when samples were ready for assay was 4 min with the sample held at 4°C. Counting of ghosts is complex, particularly when Ca-containing contracted ghosts are

TABLE III
ATPase Activity of Resealed Ghosts as a Function of the Concentration of Calcium Added to the Hemolyzing Solution*

Ca content of hemolyzing solution	‡Actual Ca content of resealed ghosts	ATPase
<i>mM</i>	<i>μmol</i> Ca/ml packed ghosts	<i>nmol</i> ³² Pi/min/10 ¹⁰ ghosts
0	0	3
0.1	—	4
0.375	0.1	8
0.75	0.57	40
1.0	0.94	46
2.5	2.80	26
5.0	5.00	24

* The hemolyzing solution also contained 2.5 mM Mg and 2.5 mM ATP. The effectiveness of resealing was such that the ATP content of sealed ghosts varied from 1.08 to 1.41 μmol ATP/ml packed ghosts.

‡ The actual calcium content of ghosts was determined experimentally from a series of parallel tubes where the Ca in the resealing mixture was labeled with ⁴⁵Ca of known specific activity, and the radioactivity of the washed ghost pellets was measured at the beginning of the incubation.

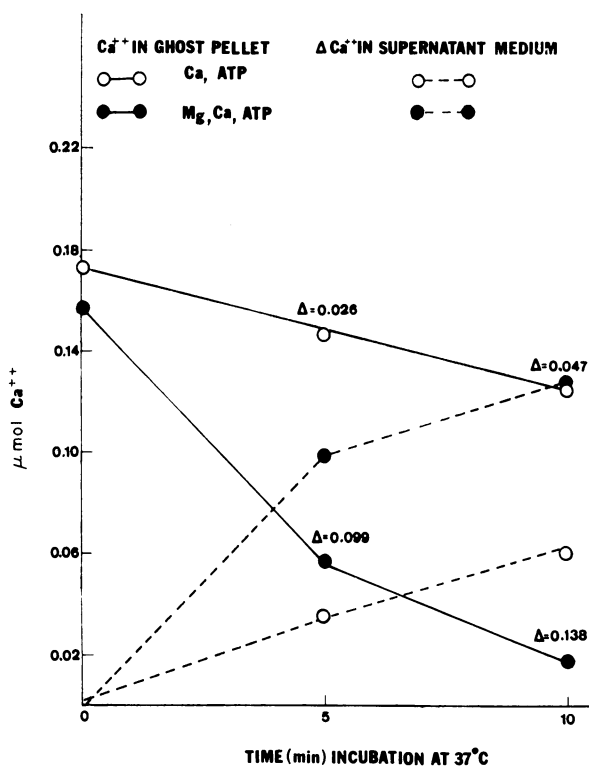


FIGURE 3 Efflux of Ca from within resealed erythrocyte ghosts incubated at 37°C. The hemolyzing solutions consisted of 2.5 mM ATP and 1 mM Ca (open circles), with the addition of 2.5 mM Mg (closed circles). The ordinate records micromoles of Ca. The solid lines refer to Ca residual in ghosts incubated under the conditions described in Fig. 2. The dotted lines indicate the appearance of Ca in the supernatant medium. The symbol Δ appearing along the course of the solid lines indicates the change in micromoles of Ca in ghosts over the given time intervals.

involved. The procedure followed is basically that described previously (25), when the ghosts were counted in the Coulter Counter with regular confirmation by hemocytometer counting with phase microscopy. Ghosts were counted at an upper threshold setting of 0-100 and a lower threshold setting of 0-5, amplification $\frac{1}{2}$, aperture current $\frac{1}{2}$, in Coulter isoton diluent with an automatic diluter. Each sample was counted three or more times until there was less than 5% variation in count. A ghost hematocrit was performed in duplicate so that a mean corpuscular volume of ghosts could be calculated. No attempt was made to standardize the hematocrit of these red ghosts. Isotopic analyses for ^{45}Ca , $[\gamma^{32}\text{P}]\text{ATP}$ and $^{32}\text{P}\text{i}$ were performed on the ghost pellet. In the experiments where Ca flux was measured by atomic absorption, the ATP content of ghosts was determined on perchloric acid filtrates by the coupled enzyme reaction utilizing glucokinase and glucose-6-phosphate dehydrogenase (19). Ruthenium red was used to inhibit the Ca, Mg-ATPase (16) and Ca efflux (26); and since it has limited capacity to penetrate plasma membranes (27), it was added during the hemolysis step at the concentrations indicated. Concentrations of ruthenium red above 120 μM interfered with the ghost-sealing procedure.

Evaluation of endocytosis. Morphologic evaluation of endocytosis was performed in each experiment by phase mi-

croscopy (7), and in specific circumstances by electron-microscopy by the procedures for fixation as previously described (7). The glutaraldehyde was prepared in pH 6.7 phosphate buffer, 45 mM with a final osmolarity of 300 mosM.

RESULTS

ATPase activity in resealed ghosts. Ca, Mg and ATP are required for endocytosis and are also the cofactors and substrate for a membrane-associated Ca,Mg-ATPase. We explored the possible relationship between ghost endocytosis and Ca,Mg-ATPase activation by comparing the optimal concentration of reactants required for each.

Measurement of ATPase activity within resealed erythrocyte ghosts is recorded in Fig. 2. Results of a single experiment typical of more than 20 are shown. The hemolyzing solution contained 1 mM Ca and 2.5 mM ATP to which $[\gamma^{32}\text{P}]\text{ATP}$ was added to provide a specific activity of 50,000 cpm/ μmol of ATP, and, where added, 2.5 mM Mg. In this experiment, the absolute amount of ATP resealed within ghosts at the beginning of the experiment (0 time) was the same whether Mg was added or omitted. After 10 min there was some decrease of $[\gamma^{32}\text{P}]\text{ATP}$ within resealed ghosts in the absence of Mg. Presumably this occurred because it was difficult to prepare ghosts totally free of Mg. The addition of Mg produced an increment in $[\gamma^{32}\text{P}]\text{ATP}$ disappearance and a similar increase in production of $^{32}\text{P}\text{i}$. The decrease in $[\gamma^{32}\text{P}]\text{ATP}$ within ghosts during the 10-min sampling period of incubation is shown in Fig. 2 as $\Delta [^{32}\text{P}]\text{ATP}$. Some of the newly generated $^{32}\text{P}\text{i}$ was found in the supernatant medium, although most was recovered within the erythrocyte ghosts during the 0-10-min incubation period. In most experiments there was good correlation between disappearance of $[\gamma^{32}\text{P}]\text{ATP}$ and appearance of $^{32}\text{P}\text{i}$. As previously noted, monitoring of the supernatant media for leak of $[\gamma^{32}\text{P}]\text{ATP}$ was performed and less than 2% of total ATP resealed within ghosts leaked into the supernatant media during the incubation. Therefore, under conditions optimal for ghost endocytosis, there is also activation of an ATPase. Since the resealing solutions were composed of choline chloride, there was no opportunity for activation of Na,K-ATPase, or for monovalent cation activation of the Ca,Mg-ATPase (11).

The Mg concentration dependence of the ATPase was measured (Table II) and occurred over a range of 0.5-2.5 mM Mg added to the hemolyzing solution mixture. As previously reported (12), optimal activation occurred when Mg and ATP concentrations were equal. Thus for a hemolyzing solution containing 1.0 mM Ca and 2.5 mM ATP, optimal ATPase activity was observed at Mg levels of 2.5 mM.

The Ca concentration dependence of the ATPase (Table III) was also determined by varying the Ca content

of the hemolyzing solution from 0.1 to 5.0 mM. The actual Ca content of the resealed ghosts at the beginning of the incubation period is also shown in Table III. There was a biphasic response of ATPase activity to an increase in the Ca content in the hemolyzing solution. Ca concentrations from 0.25 mM to 1.0 mM produced activation, while concentrations of Ca in excess of 2.5 mM produced inhibition. A single experiment typical of three is shown. The concentrations of Mg and ATP used to study this biphasic effect of Ca were those previously shown to provide optimal ghost endocytosis (9), although given the complex interaction of these three reactants, other results could have been obtained if we had also varied the Mg and ATP concentrations.

The ATPase under study in the resealed ghosts required Mg (Table II) and manifested a 10–12-fold increase in activity with increasing concentrations of Ca up to 1.0 mM (Table III). These are the characteristics of a Ca,Mg-ATPase known to be present at the inner surface of the erythrocyte plasma membrane (11, 12). Therefore endocytosis and Ca,Mg-ATPase activity share a requirement for Mg (Table II) (9) and both phenomena are dependent on the concentration of Ca added to the resealing solution, with stimulation of each occurring at Ca concentrations up to 1.0 mM and inhibition of each occurring at Ca concentrations above 2.5 mM (9) (Table III).

Calcium efflux from resealed ghosts. The requirements for optimum ghost endocytosis are also similar to the conditions described for Ca efflux from resealed ghosts (12). In the experiment measuring Ca efflux in Fig. 3, there was no Ca added to the external medium and the Ca resealed within erythrocyte ghosts was labeled with ^{45}Ca so that the specific activity was approximately 10^8 cpm/ μmol Ca. Calculation of Ca efflux could be obtained by measuring either the disappearance of ^{45}Ca from the centrifuged ghost pellet or the appearance of ^{45}Ca in the supernatant medium, and both measurements were routinely used to check on the accuracy of recovery of ghosts and supernatant medium (Fig. 3). Addition of 2.5 mM Mg led to a threefold or greater increase in Ca efflux and this result therefore paralleled the increase in ATPase activity that also occurred upon Mg addition. These results are consistent with previous proposals linking Ca efflux in erythrocytes and their ghosts to a Ca,Mg-ATPase (12–14, 21).

Flux measurements with radioisotopes have the advantage of simplicity and ease of analysis; however, they are subject to a variety of sources of error. Therefore, Ca efflux experiments were repeated with atomic absorption spectrophotometry to measure actual Ca content within ghosts at the end of the incubation period. Three experiments were performed wherein atomic absorption was used in parallel with measurements of

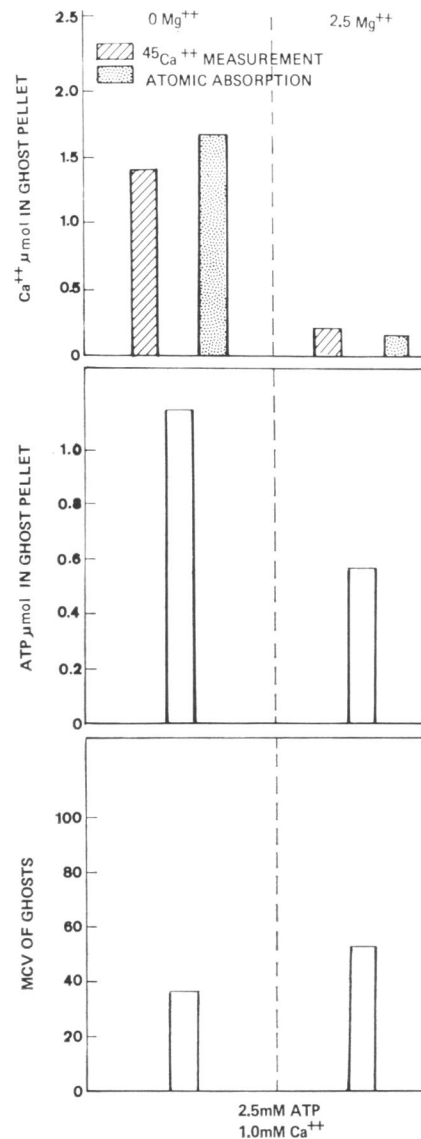


FIGURE 4 Ca movements were measured by atomic absorption spectrophotometry and radioisotopically. The hemolyzing solutions contained 1.0 mM Ca and 2.5 mM ATP without (left side) and with (right side) the addition of 2.5 mM Mg. Since relatively large samples are required for atomic absorption measurements, 2 ml of packed ghosts were used. The results in the figure are those recorded per 2 ml packed ghosts after the equilibration, resealing, washing, and subsequent incubation at 37°C for 15 min. MCV, mean corpuscular volume.

^{45}Ca flux. In the single experiment shown in Fig. 4, Ca disappearance from ghosts was similar whether measured by atomic absorption or by determination of ^{45}Ca isotopic activity. Furthermore, after the incubation period, there was a distinct decrease of ATP in the Mg-containing resealed ghost pellet. The conditions in these experiments were parallel to those described above,

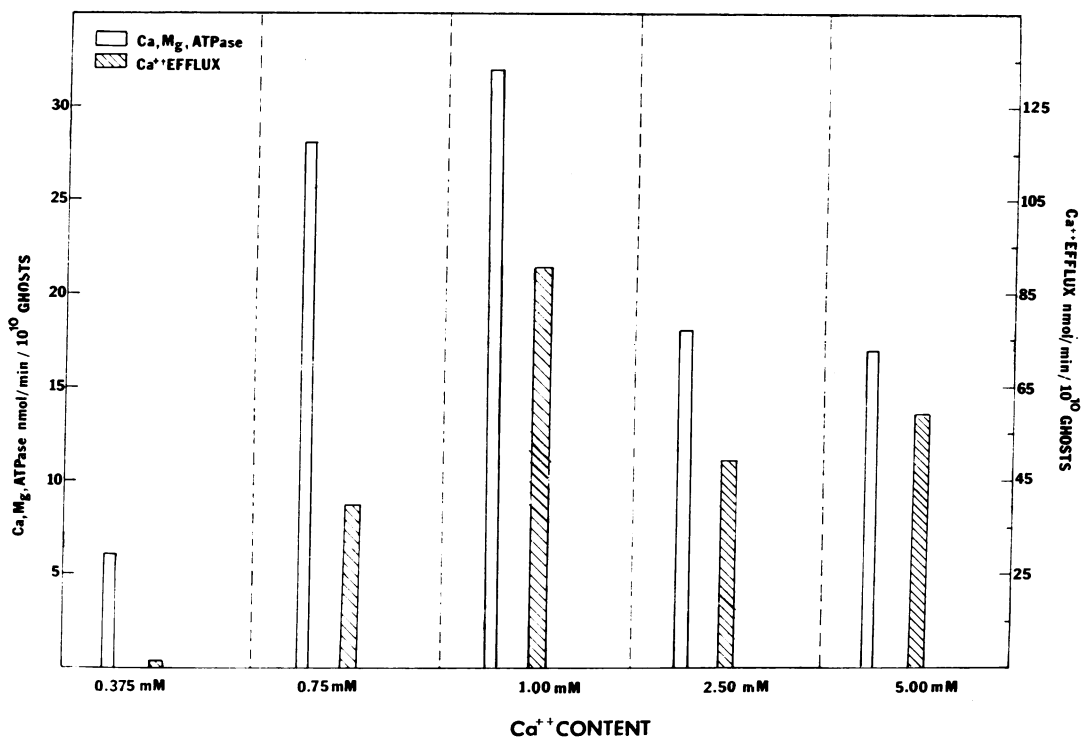


FIGURE 5 Ca,Mg-ATPase and Ca efflux as a function of the external Ca concentration of the hemolyzing solution. The Ca content of the hemolyzing solution is recorded on the abscissa for each panel. The left ordinate indicates the Ca,Mg-ATPase activity (open bars) whereas the right ordinate indicates Ca efflux from ghosts (hatched bars).

where it could be shown that less than 2% of resealed ATP leaked from ghosts during the incubation period. Therefore it was unlikely that the ATP disappearance from ghosts represented ATP leak and more likely that ATP hydrolysis accounted for the decrease in enzymatically measurable ATP. These studies showed that upon addition of Mg to the resealing solution there was a substantial movement of Ca out of ghosts concomitant with a reduction of ATP content of ghosts, thus confirming by alternative methods the results obtained with ⁴⁵Ca and [³²P]ATP. It is of interest that part of the sharp reduction of mean corpuscular volume seen with ghosts incubated in Ca alone is blocked by the simultaneous addition of Mg.

Ca efflux as a function of the varying concentration of Ca in the hemolyzing solution was then determined. An experiment is shown in Fig. 5 where Ca,Mg-ATPase activity and Ca efflux were measured as a function of the Ca content of the hemolyzing solution. Maximal activation of both Ca efflux and Ca,Mg-ATPase occurred at Ca concentrations of 0.75–1.00 mM with a biphasic aspect to the curve. Thus the three phenomena under study, endocytosis, Ca,Mg-ATPase, and Ca efflux, share a requirement for Mg and show nearly identical

responses to addition of Ca over a concentration range of approximately 0.25–5.0 mM.

17 experiments were performed in which Ca efflux was measured in parallel with Ca,Mg-ATPase activity at optimal conditions for each. These values were used to calculate a Ca/P ratio that represents the number of moles of Ca transferred out of ghosts per mole of ATP hydrolyzed to ADP. The mean±SD Ca/P ratio calculated was 1.24±0.48 for 17 determinations, and this value is in substantial agreement with values of 0.856, 1.27, and 1.39 recently reported by Schatzmann (21).

Morphologic appearance of endocytosis in relation to the activation of Ca,Mg-ATPase and to Ca efflux (Table IV, Fig. 6). Experiments were designed to measure Ca,Mg-ATPase and Ca efflux simultaneously while monitoring endocytosis by electron microscopy. Interpretation of the onset and extent of endocytosis was made independently on duplicate coded samples. In the absence of added Mg, endocytosis did not occur (7, 9) and addition of Ca and ATP without Mg produced constricted ghosts (28–30) (Fig. 6A) with bell-like projections. Addition of Mg resulted in the activation of Ca,Mg-ATPase and efflux of Ca from within re-

sealed ghosts (Table IV) with a Ca/P ratio in this experiment of 0.95 (Table IV). However, despite the fact that the Ca,Mg-ATPase was activated and Ca was being extruded (Table IV), there was little morphologic alteration for 7 min (Fig. 6B, C, and D). Ca,Mg-ATPase activity was relatively stable over the incubation period at 44–50 nmol ³²Pi/min per 10¹⁰ ghosts. Ca efflux was stable for the first 7 min and paralleled Ca,Mg-ATPase activity at approximately 46 nmol Ca/min per 10¹⁰ ghosts. During the last 8 min of incubation Ca efflux declined to a value of 28, perhaps as a consequence of a decreased intracellular Ca content available for efflux. As a consequence of decreasing content of intra-ghost Ca, the activity of the Ca,Mg-ATPase should have also decreased (Fig. 5). It may be that an amount of Ca sufficient to continue to stimulate the Ca,Mg-ATPase for an additional 8 min had been bound to rate-controlling sites of the enzyme at the inner wall of the erythrocyte membrane. However, samples taken for electron microscopy at 0, 3, and 7 min in this and two other experiments never revealed endocytosis, while there was evidence of Ca,Mg-ATPase activity and Ca efflux both occurring optimally during this initial 7-min of incubation. Endocytosis appeared after 15 min of incubation in Mg-containing ghosts (compare Fig. 6A and E) and then in almost every ghost (Fig. 6E, and F). By the time endocytosis occurred, the Ca-induced constriction had essentially disappeared. This lag between extensive extrusion of Ca and continuous Ca,Mg-ATPase action on the one hand and endocytosis on the other was reproducible in two other experiments. In an additional experiment, samples were taken at 0, 3, 8, 10, 13, and 16 min and showed a gradual increase in endocytosis from 10 to 16 min, but no endocytosis before 7 min.

Inhibition of endocytosis (Fig. 7, Table V). It was then important to determine if an inhibitor of the Ca transport system or of Ca,Mg-ATPase would also block endocytosis. Ruthenium red has been reported to be both an inhibitor of Ca transfer (26) and of Ca,Mg-ATPase (16). Accordingly, ruthenium red was resealed within erythrocyte ghosts and it was determined in four experiments that there was a log concentration-dependent inhibition of both Ca efflux and Ca,Mg-ATPase with 50% inhibition of each function occurring at a ruthenium red concentration in the hemolyzing solution of approximately 60–80 μ M.

Coded samples were prepared for electron microscopy and the interpretations were rendered independently by two observers. It can be seen (Fig. 7, Table V) that inhibition of Ca,Mg-ATPase and Ca efflux by ruthenium red was accompanied by extensive inhibition of endocytosis. When 100 μ M ruthenium red was used, endocytosis was totally inhibited while there was still

TABLE IV
ATPase and Ca Efflux Concomitants of Endocytosis

Time	ATP content of ghosts	Ca content of ghosts
<i>min</i>	<i>nmol/ml packed ghosts</i>	<i>nmol/ml packed ghosts</i>
0	2,080	605
3	1,930	426
7	1,740	281
15	1,390	58

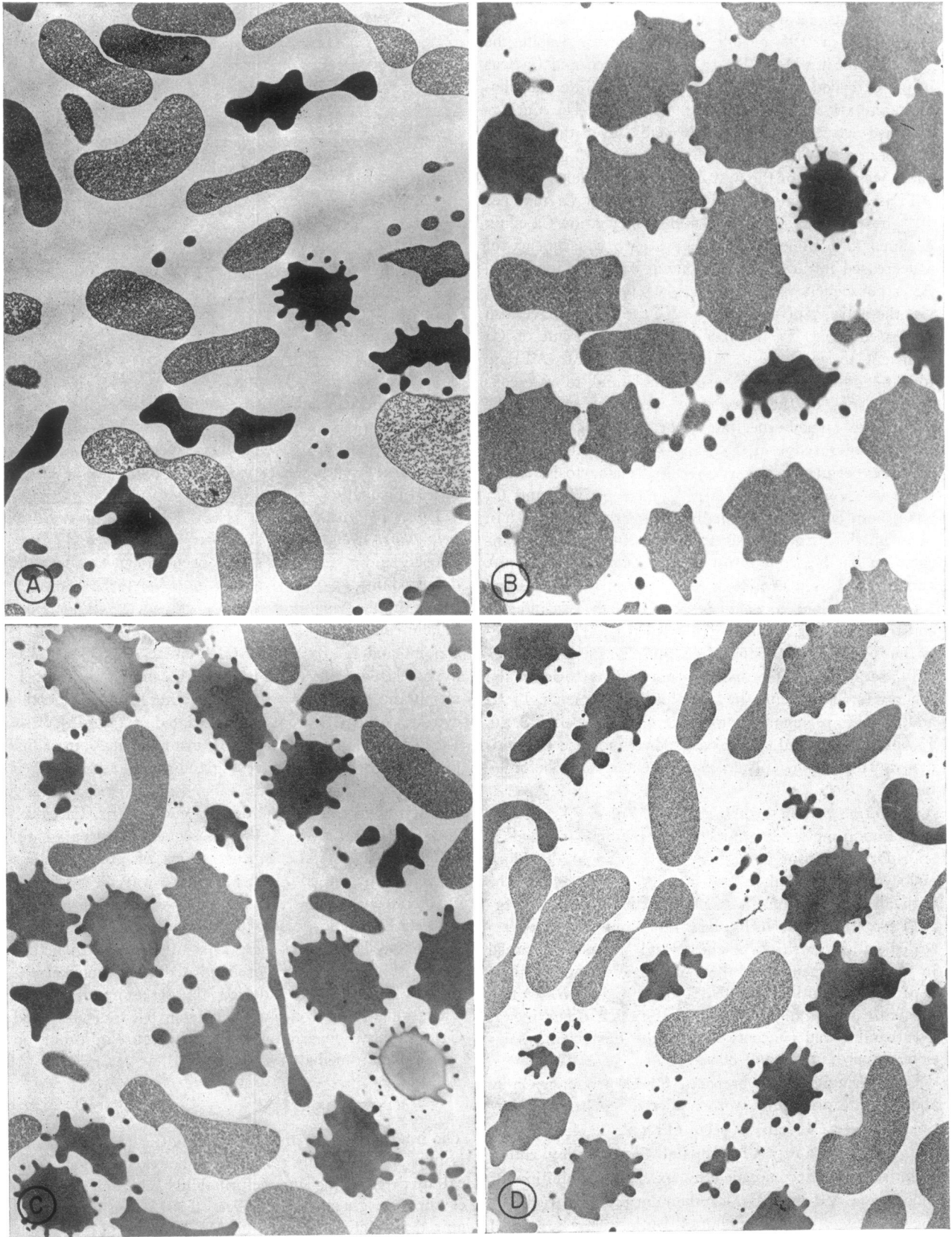
The resealing solution consisted of 2.5 mM ATP, 1.0 mM Ca, and 2.5 mM Mg.

substantial residual activity of Ca,Mg-ATPase and its linked Ca efflux. The value for the residual Ca in the 35- μ M ruthenium red sample (Table V) was lower than that for the parallel control sample because the resealing of Ca was somewhat lower in the 35- μ M ruthenium red sample at zero time in this experiment but the Ca efflux rate was nevertheless linear over the initial 7 min of incubation.

Effect of prostaglandins (PG) E₁ and E₂ on erythrocyte ghost endocytosis, Ca efflux, and Ca,Mg-ATPase. Prostaglandins E₁ and E₂ have been shown to affect the deformability characteristics of intact erythrocytes (31). Since the deformability of the erythrocyte is under metabolic control, represented in part by erythrocyte ATP content and in part by its Ca content (32, 33) it was hypothesized that PGE₁ and PGE₂ might exert their membrane effects by perturbing either Ca efflux, endocytosis, or the membrane-associated Ca,Mg-ATPase. We attempted to determine if prior incubation of intact erythrocytes with prostaglandins E₁ and E₂ or if addition of prostaglandins E₁ and E₂ to the resealing solution altered the capacities for endocytosis, Ca efflux, and Ca,Mg-ATPase of the subsequently prepared resealed ghosts. At the concentrations of prostaglandins E₁ and E₂ first used (10⁻¹⁰ M), there was no consistent effect on endocytosis, Ca efflux, or activity of Ca,Mg-ATPase. Altering prostaglandin concentration to 10⁻⁹ or 10⁻¹¹ M produced no change. This situation held whether the prostaglandins were preincubated with intact erythrocytes or were added during the resealing of ghosts. Variation of Mg concentration from 0 to 2.5 mM and Ca concentration from 0 to 1.0 mM brought out no prostaglandin-mediated effect.

DISCUSSION

The interplay of the three reactants, Ca, Mg, and ATP, is important not only for ghost endocytosis but is critical in determining the deformability characteristics of erythrocyte ghosts (33). These three materials are required for activation of a Ca,Mg-ATPase (11–13) and



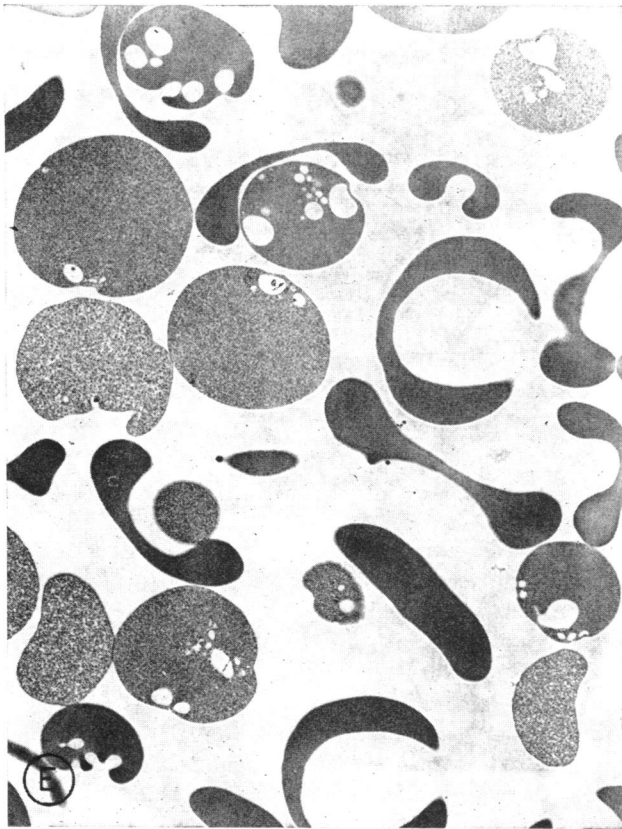


FIGURE 6 Electron microphotographs of resealed ghosts. A. Ghosts were resealed with 2.5 mM ATP and 1.0 mM Ca and then incubated for 15 min at 37°C. B, C, D, E, and F are electron microphotographs of ghosts hemolyzed with 2.5 mM ATP, 1.0 mM Ca, and 2.5 mM Mg and then incubated at 37°C for 0 min (B), 3 min (C), 7 min (D), and 15 min (E and F). Magnification of A-E is 4,800 \times . The magnification of F is 1,900 \times . Because of the short hemolysis time used there are some incompletely hemolyzed cells.

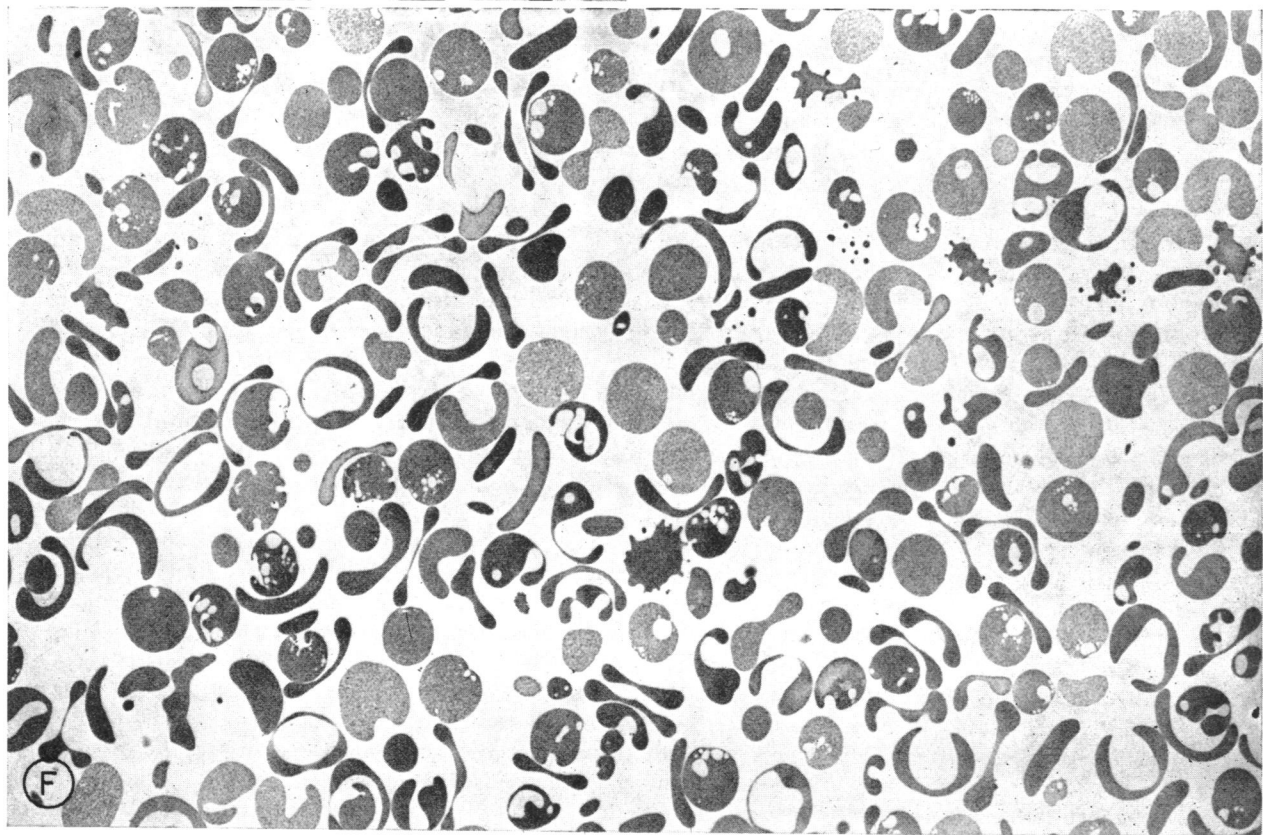


TABLE V
Effect of Ruthenium Red on Ca, Mg-ATPase, Ca Efflux, and Endocytosis

	Ca, Mg-ATPase		Ca efflux‡		Ca content of ghosts after 15 min of incubation	Endocytosis score§
	*	%	%		nmol/10 ¹⁰ ghosts	
Control	61	100	43	100	69	105
35 μ M ruthenium red	37	60	33	76	51	27
70 μ M ruthenium red	27	44	26	60	159	8
100 μ M ruthenium red	19	31	20	46	168	0

* Units same as Table II for ATPase.

‡ Efflux rate was calculated during the first 3 min of incubation at 37°C where the rate was linear with respect to time and is recorded as nanomoles of Ca leaving ghosts per minute per 10¹⁰ ghosts.

§ A rough approximation of endocytosis was made by counting endocytic vacuoles in resealed ghosts in fields of comparable erythrocyte density on four photographic prints of different fields.

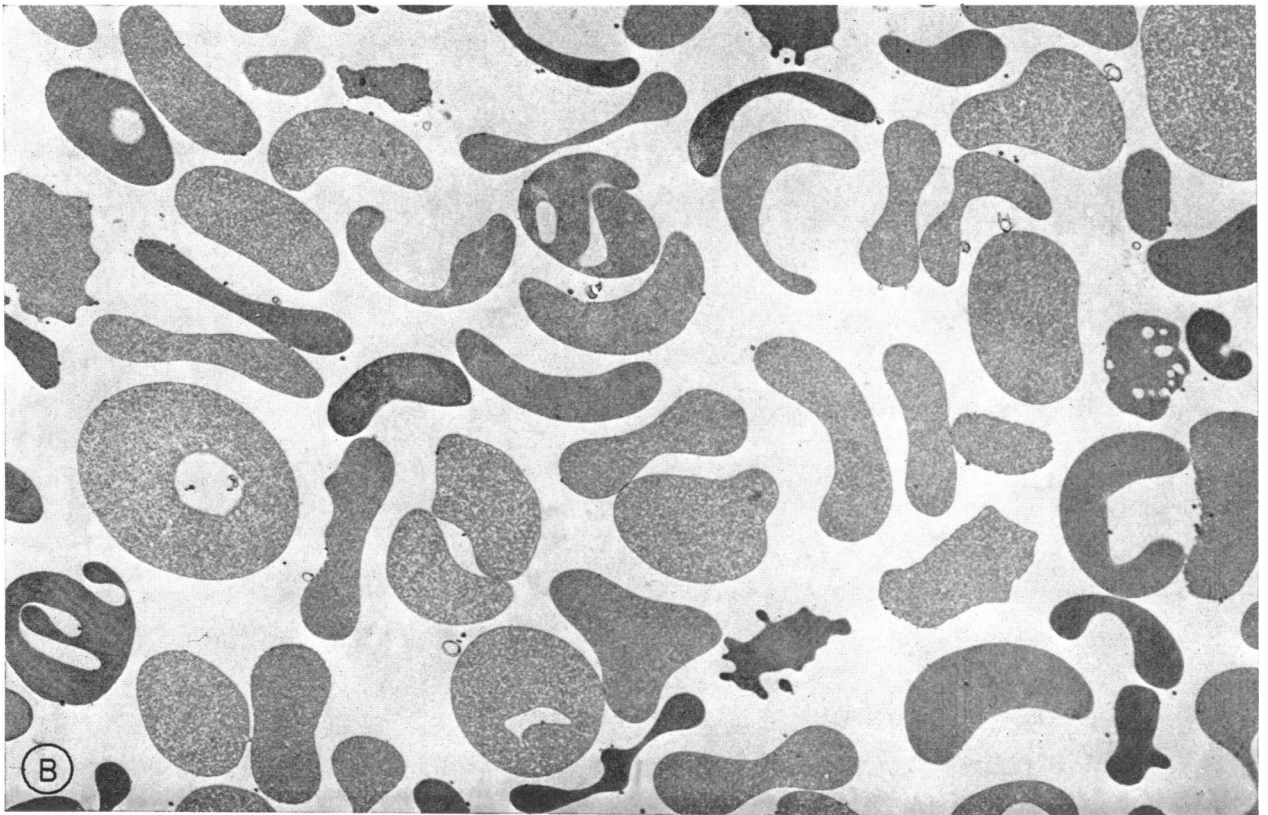
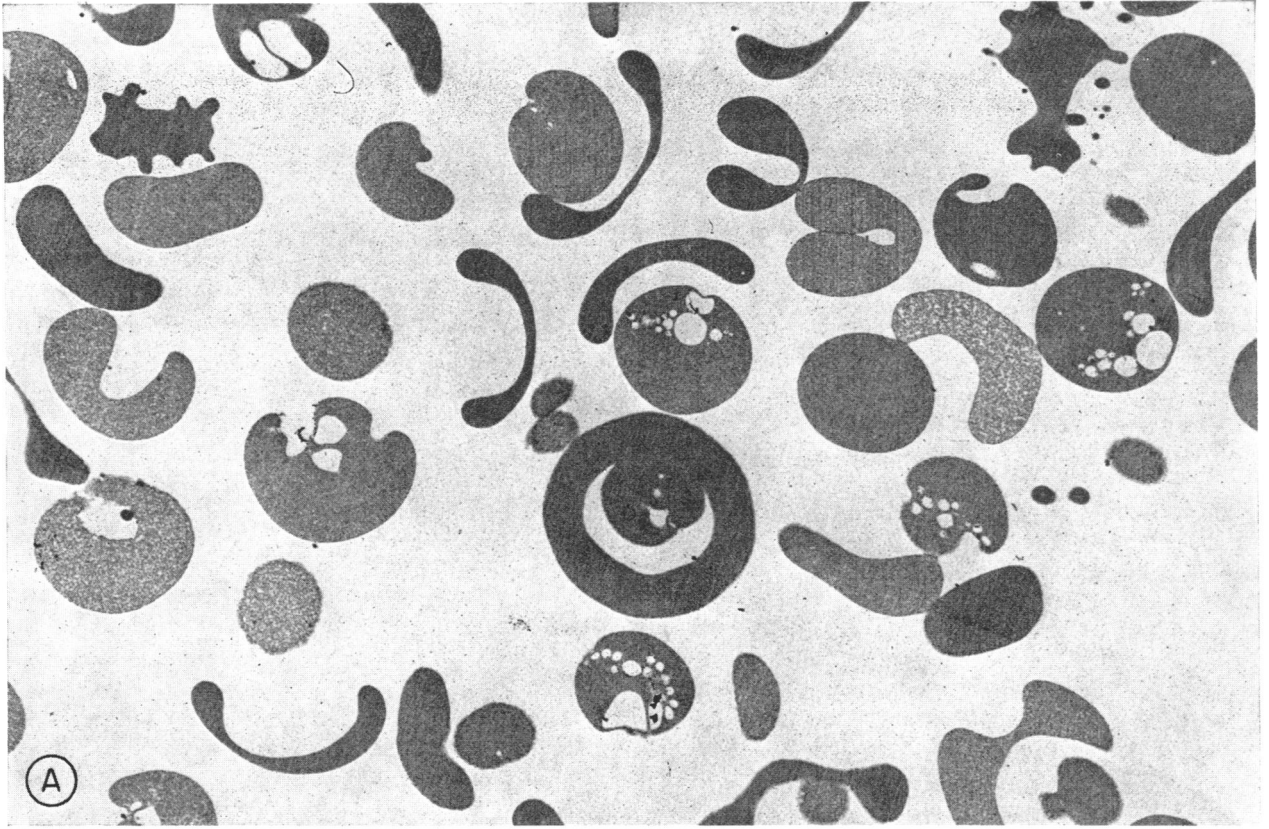
for the linked unidirectional efflux of Ca from within resealed ghosts (12–14). The evidence relating erythrocyte ghost Ca, Mg-ATPase and Ca efflux is by now quite compelling (21). Because of the similarity in required reactants and because of the similar biphasic response of endocytosis, Ca, Mg-ATPase, and Ca efflux to increasing concentration of Ca (11), we explored the possibility that the Ca, Mg-ATPase and the efflux of Ca might have an important role in energized ghost endocytosis.

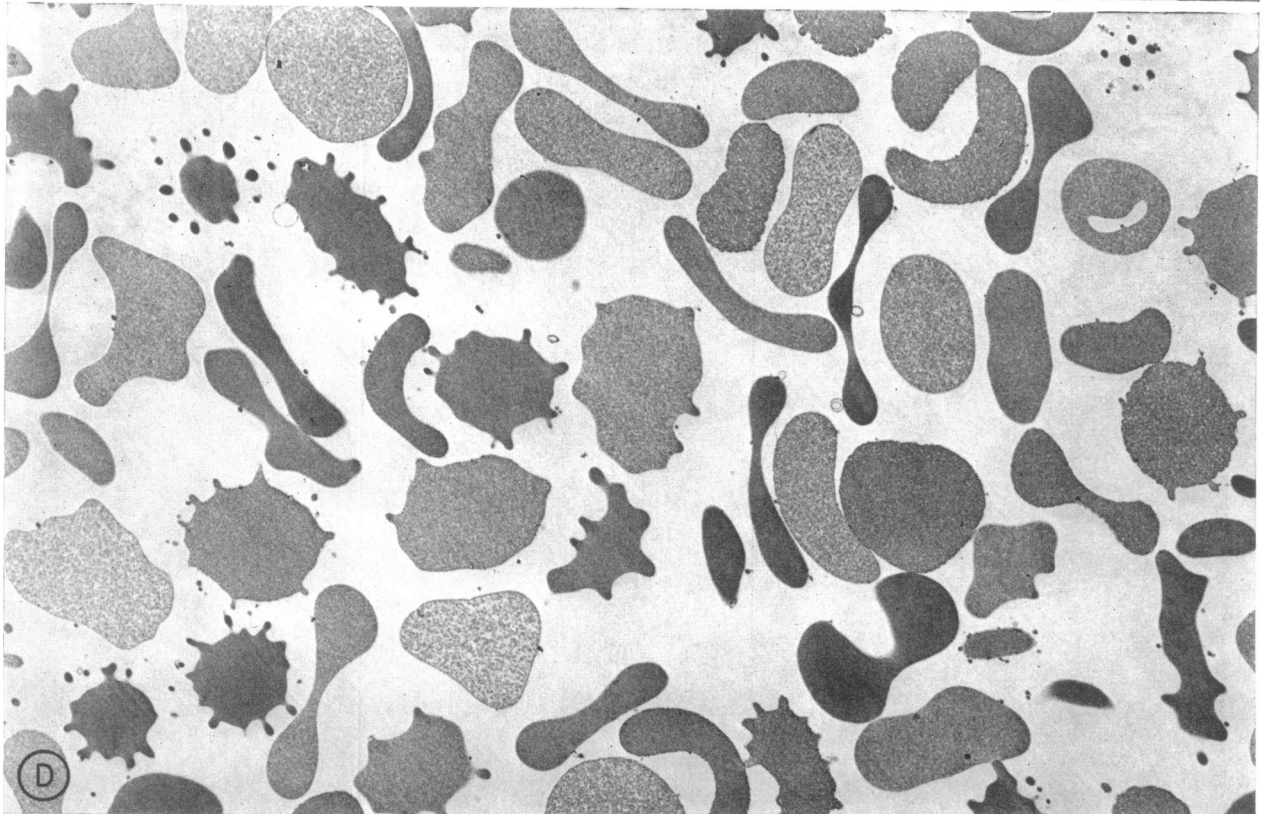
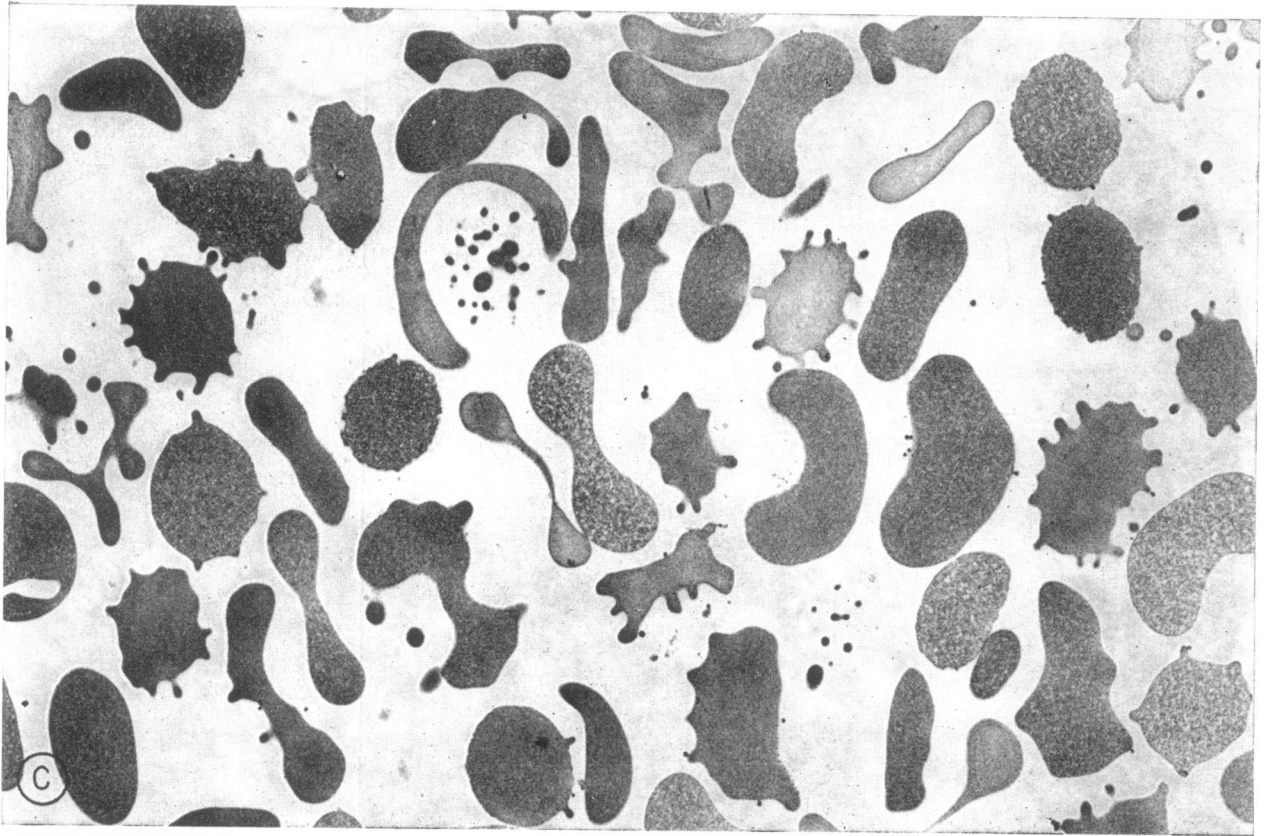
In the experiments reported now it appears that the same strict requirement for Mg exists for activation of Ca, Mg-ATPase, Ca efflux, and endocytosis (Table II, Fig. 3). Furthermore, the biphasic response to increasing Ca content in the resealing mixture is essentially identical for optimal endocytosis (9), Ca, Mg-ATPase activity, and Ca efflux (Fig. 5).

In pursuing the apparent parallelism between endocytosis, Ca, Mg-ATPase, and Ca efflux we studied the role of ruthenium red. Ruthenium red is reported to be an effective inhibitor of the Ca, Mg-ATPase of erythrocyte ghosts (16), and we confirmed this observation (Table V). The 50% inhibition value obtained in our study is 60–80 μ M, substantially greater than the value of 10 μ M previously reported (16). However, in that study unsealed membranes were used, whereas we studied resealed ghosts (Methods), where it could be anticipated that problems of continued access of inhibitor molecules to critical sites would arise. Ruthenium red also inhibits mitochondrial Ca transport (26) and effectively blocked Ca efflux from resealed ghosts (Table V). There was almost parallel ruthenium red inhibition of Ca, Mg-ATPase and Ca efflux and it was not possible to determine from this sort of analysis which, if either, was the prior event. Of critical importance was the sensitivity of endocytosis to ruthenium red inhibition (Fig. 7). In fact ruthenium red totally blocked endo-

cytosis at concentrations that left 30–40% residual Ca, Mg-ATPase activity linked to Ca efflux (Table V). It is possible that ruthenium red combines with a membrane component which may be of major importance in energized endocytosis and which may also serve to link Ca, Mg-ATPase with a Ca transposing or translocating system. The studies of Luft (27) have shown that ruthenium red appears to bind to acid mucopolysaccharides and is generally excluded by plasma membranes. When incubated with intact erythrocytes, it can be shown to stain a thin layer of the plasma membrane with a pattern showing some extension into the membrane substance proper, as though there were a degree of porosity. Under certain circumstances ruthenium red may also bind to lipid, particularly acid phospholipids, so that membrane components susceptible to ruthenium red would include lipids as well as glycoproteins. Of interest is that ruthenium red seems to localize at sites where mechanical forces are proven or suspected, as along collagen fibers and particularly at the myotendinal junction. Thus the observation that ruthenium red appears to be a more potent inhibitor of endocytosis than Ca efflux or Ca, Mg-ATPase suggests that the inhibitor may attack a membrane component which is involved in the mechanical aspects of endocytosis and which may also link the ATP-hydrolyzing system with a Ca-translocating system.

The biphasic response of the three phenomena under consideration to variations in Ca concentration is of interest, with the response changing from stimulation to inhibition over a concentration range of 0.1–2.0 mM (9) (Fig. 5). It is conceivable that a single class of molecule responds to increases in Ca concentration in a biphasic manner. Thus it has been observed (11) that increasing concentrations of Ca interfere with the binding of required Mg, thereby producing inhibition of Ca, Mg-ATPase. However, the actions of calcium are





complex at concentrations greater than 1 mM and it also has the capacity to aggregate and otherwise alter erythrocyte and other membrane proteins (34, 35). Therefore certain concentrations of Ca might by action on one class of membrane component activate endocytosis, Ca efflux, and Ca,Mg-ATPase, whereas higher concentrations of Ca could precipitate other specific membrane proteins (35, 36), thus producing rigidity and perhaps loss of function that would now interfere with the mechanical aspects of endocytosis, Ca,Mg-ATPase, and Ca efflux.

While there is substantial similarity between endocytosis and Ca efflux and Ca,Mg-ATPase, there are differences. It was reported that efflux of Ca from resealed ghosts could be energized equally well by ATP, CTP, and UTP (14), whereas CTP and UTP were less than one third as effective as ATP in energizing ghost endocytosis (9).

Study of the possible role of prostaglandin E₁ and E₂ was prompted by their reported ability to alter erythrocyte membrane deformability (31) when Ca is added to the suspending media (36). Our studies revealed no effect of prostaglandin E₁ and E₂ on endocytosis, Ca efflux, or Ca,Mg-ATPase, in the concentration range that produces (36) small but highly reproducible membrane alterations.

If endocytosis were a direct reflection of Ca,Mg-ATPase activation or of Ca extrusion, we would have expected to see endocytic vacuoles forming immediately upon resealing and incubation at 37°C. However, while Ca,Mg-ATPase was steadily active over a 15-min period (Table IV) (Fig. 6) and optimal Ca efflux occurred over an initial 7-min period (Table IV), no endocytosis was seen until more than 7 min had elapsed. The lag between Ca efflux and Ca,Mg-ATPase on one hand and endocytosis on the other suggests that a slower mechanochemical event could have preceded endocytosis.

Alternatively, it could be proposed that the time lag served only to displace enough Ca from the ghost membrane so that Mg-ATP could produce endocytosis. From the data in Table IV it could be argued that when the ghost Ca content dropped below 0.281 $\mu\text{mol/ml}$, endocytosis could occur, and therefore, the optimal Ca concentrations for endocytosis are lower than those for Ca efflux. Ghost Ca contents of 0.28 $\mu\text{mol/ml}$ or less can be achieved by hemolyzing ghosts in the presence of approximately 0.5 mM Ca (Table Ic and Table III) or less; however, such conditions result in decreasing endocytosis, paralleling the decrease in Ca concentration (9). Furthermore, it can be seen in Table V that

use of 35 μM ruthenium red only modestly impaired Ca efflux and that after 15 min of incubation, the Ca content of the ghosts so treated was similar to that of the control; however, despite the low Ca content, endocytosis was distinctly impaired. Therefore the absolute level of Ca in ghosts appears to exert some control over the extent of endocytosis, which, however, seems to relate more directly to the extent of ATPase activity and Ca translocation.

Our morphologic studies (Fig. 6) confirm the previously described Ca-linked constriction of erythrocyte ghosts (28-30). In the absence of added Mg, ghosts containing Ca and ATP remained in a sharply constricted form for at least 15 min of incubation at 37°C (Fig. 6A). However, upon addition of Mg, which activates the Ca,Mg-ATPase and Ca efflux (Fig. 6E and Table IV), there is distinct loss of ghost constriction, which is time-dependent and coincides with the efflux of most of the Ca from ghosts. In contrast to endocytosis, ghost constriction appears to relate almost directly to the absolute amount of intra-ghost Ca present at a given time. When 35 μM ruthenium red was used (Table V, Fig. 7B), there was impaired endocytosis, but Ca efflux was well enough preserved to result in a sharp reduction of intracellular Ca in 15 min and a substantial reversal of constricted ghost forms. Therefore, the proposed Ca-activated contractile protein is not extensively affected by ruthenium red.

Ghost endocytosis, with its dependence on Ca, Mg, and ATP and its apparent relationship to Ca,Mg-ATPase, and Ca translocation, seems to be similar to the more general phenomenon of cell membrane fusion (37). In a recent review Poste and Allison (37) note that a variety of forms of cell membrane fusion, including pinocytosis, release of secretory granules, and virus-induced cell fusion, have similar biochemical characteristics, consisting of Ca,Mg-ATPase activation, parallel displacement of Ca, and inhibition of the phenomenon by excess Ca.

If endocytosis is thought of as simplistically consisting of a membrane invagination followed by contraction of the mouth of the invagination and subsequent membrane resealing (1, 2), one could hypothesize that the initial membrane expansion and inward buckling involves a degree of membrane deformability associated with Mg and ATP (33), that the contraction of the mouth of the invaginated membrane segment is perhaps mediated by a Ca-stimulated contractile protein (thus accounting in part for the Ca enhancement of endocytosis), and that the fusion of the endocytic vacuole is

FIGURE 7 Electron microphotographs of erythrocyte ghosts hemolyzed with 2.5 mM ATP, 1.0 mM Ca, and 2.5 mM Mg and then incubated for 15 min at 37°C. No ruthenium red was added in A, 35 μM ruthenium red was added to the hemolyzing solution of B, 70 μM ruthenium red to C, and 100 μM ruthenium red to D. Magnification in all is 4,900 \times .

controlled by mechanisms related to Ca,Mg-ATPase and Ca translocation, as are other examples of plasma membrane fusion (37).

ACKNOWLEDGMENTS

This project was supported by National Institutes of Health Grant S RO1 AM 13682.

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