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ENERGY-DEPENDENT ENDOCYTOSIS IN ERYTHROCYTE GHOSTS

IV. EFFECTS OF Ca^{2+}, Na^+, K^+, AND 5'-ADENYLYLIMIDODIPHOSPHATE

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SUMMARY

The requirement of actual splitting of ATP for endocytosis in erythrocyte ghosts has been confirmed by use of the ATP analog, 5'-adenylylimidodiphosphate, (AMP-P(NH)P). This compound, in which the oxygen connecting the β and γ phosphorus atoms was replaced by an NH group, did not cause endocytosis nor was it a substrate for ATPase activity. AMP-P(NH)P was a competitive inhibitor both for the endocytosis and the Mg^{2+}-ATPase activities. The K_i of AMP-P(NH)P for Mg^{2+}-ATPase activity was $2.0 \cdot 10^{-4}$ M and, while the K_m of ATP for this activity was also $2.0 \cdot 10^{-4}$ M indicating nearly identical affinities of ATP and AMP-P(NH)P for the active site. ADP, or ADP plus orthophosphate, did not cause endocytosis, showing that endocytosis was not due to binding of the products of ATP hydrolysis. Sodium or potassium ion or ouabain had no effect on endocytosis, which eliminated the possibility of involvement of the Na^+, K^+ ATPase in the endocytosis process. Calcium could not be substituted for magnesium; rather it inhibited endocytosis at the concentration of $1 \cdot 10^{-3}$ M. EGTA relieved the inhibitory effect of Ca, which indicated that the binding of calcium to the membrane was reversible. These experimental results reaffirm the conclusion that ATP must be split to engender endocytosis under these conditions. Some characteristic parameters of the hemoglobin-free porcine erythrocyte ghosts were studied in order to characterize the system more adequately.

INTRODUCTION

It has become quite clear that erythrocyte ghosts carry out endocytosis in the presence of ATP [1–6]. Although the physiological significance of this phenomenon

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Abbreviations: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; AMP-P(NH)P, 5'-adenylylimidophosphate; EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*'-tetraacetic acid.

is not clear, vacuoles have also been reported in circulating red cells of splenectomized patients [7]. ATP is required to maintain the discoid shape of the whole erythrocyte [8–10] and to cause endocytosis both in the ghosts and in whole cells. This suggests that there must be a specific system on the erythrocyte membrane in order to carry out these configurational changes.

In the previous reports, we showed that ATP and magnesium ion were required for endocytosis [1, 3] and that both ATPase activity and endocytosis activity were similarly inhibited by various maleimide compounds [11]. It has been predicted that the magnesium stimulated ATPase activity might be closely related to the performance of endocytosis [1–4]. Although the requirement for ATP was well demonstrated, there still remained many questions concerning the relationship between the presence of ATP and the actual event of endocytosis. Much of our research efforts have been directed toward gaining a better understanding of the mechanism of endocytosis in ghosts. In this report we present more direct evidence of the requirements for actual ATP splitting for endocytosis.

METHODS AND MATERIALS

Porcine erythrocyte membranes were prepared using fresh blood obtained from the slaughter house immediately after slaughtering. The method of Dodge et al. [12], with 1 mM EDTA added to all the buffers [11], was used for isolation of ghosts. Packed ghosts, after final washing by cold Tris–HCl (28 mM Tris, 1 mM EDTA, pH at 4 °C, 7.4), were assayed for protein concentration. They were then rewashed in 10 volumes of 50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid titrated by triethanolamine to pH 7.4 at 4 °C (TES–triethanolamine [3]. After washing the ghosts were suspended in 50 mM TES–triethanolamine buffer at a protein concentration of 5 mg per ml. Since Tris–HCl has a low buffer capacity at pH 7.4, and a large temperature dependent pH variation, TES–triethanolamine buffer was selected for these experiments. Since the triethanolamine interfered with the protein concentration assay by the biuret method [13] or the Lowry method [14], it was necessary to measure the protein concentration before suspending in TES–triethanolamine buffer.

Hemoglobin content was measured by the method of Dodge et al. [12]; crystalline human hemoglobin was used as a standard. Total phospholipid phosphorous was determined by perchloric acid digestion of ghosts as described by Chen et al. [15] followed by an inorganic phosphate assay by the method of Berenblum and Chain as modified by Martin and Doty [16]. The total weight of phospholipid was calculated using the assumption of an average phospholipid molecular weight of 750. Sialic acid was determined by the method of Warren [17] after digestion by neuraminidase (Sigma type IV) or acid hydrolysis in 0.05 M H_2SO_4 at 80 °C for one h. *N*-Acetylneuraminic acid (Sigma type IV) and bovine submaxillary mucin (Worthington Biochemical Co.) were used as standards.

ATPase activity was measured as follows: each experiment was done in duplicate or triplicate. The reaction mixture was adjusted to a volume of 0.9 ml which contained the buffer, metal ions, inhibitor where used and 1 mg of ghost protein.

The mixture was preincubated for 5 min at 37 °C prior to starting the reaction by addition of 0.1 ml of a solution which was 30 mM in ATP and in which $Tris^+$ was the only counter-ion (Tris–ATP). After 30 min incubation the reaction was stopped by addition of 2.5 ml cold diluted silicotungstic acid and the amount of inorganic phosphate released was measured [16]. ATPase activities were measured in the presence of 3 mM $MgCl_2$ and 50 mM triethanolamine–TES buffer, pH 7.4 at 37 °C. For assay of ATPase activity in the presence of other metal ions, the following concentrations of salts were normally used; 20 mM KCl, 100 mM NaCl and 0.5 mM $CaCl_2$. Except for the data presented in Table I, ATPase activity was measured in the presence of 1 mM primaquine hydrochloride. Primaquine hydrochloride was prepared from the phosphate form (Sigma). The stock calcium solution was made from $CaCO_3$ by titration with HCl to neutral pH. Tris–ATP solution was prepared from the disodium salt form of ATP (Sigma type V) by passing it through an ion exchange column which had been pre-equilibrated with Tris–HCl (Dowex 50w-X8, 1 cm × 20 cm). Endocytosis activity was expressed as % of ghosts in the vacuolated form, as determined by the method previously described [11]. The same system as was used for the ATPase assay was also used for the corresponding endocytosis assays.

P-Nitrophenylphosphate hydrolysing activity [18] was measured as follows: 50 μmoles of buffer (MES–NaOH, pH 5.0 at 37 °C or TES–triethanolamine at 37 °C pH 7.0); 5 μmoles of $MgCl_2$; 20 μmoles of KCl when needed and 1 mg of ghost protein were made up to 0.9 ml. After preincubation for 5 min, the reaction was started by addition of 0.1 ml of 0.1 M *p*-nitrophenylphosphate (Aldrich Chemicals) and incubated 30 min at 37 °C. One ml of cold 6 % perchloric acid was added to stop the reaction and the resulting mixture was spun in a clinical centrifuge to remove the protein. One ml of the supernatant was taken and mixed with 3 ml of 0.1 M NaOH, and the absorbance was read at 410 nm. Nitrophenol solution (10 nmoles per ml; from Sigma Chemical) was used for the standard. Acetylthiocholine esterase activity was determined by the method of Ellman [19]. Polyacrylamide gel electrophoresis was carried out according to the method of Neville [20], using the following gels and buffers: The upper reservoir buffer contained 0.04 M boric acid, 0.041 M Tris, pH 8.64 and was 0.1 % in sodium dodecylsulfate; the upper gel was 3 % acrylamide in 0.541 M Tris–H_2SO_4, pH 6.1, crosslinked by 0.2 % bis-acrylamide; the lower gel was 11 % acrylamide in 0.4244 M Tris–HCl, pH 9.50, crosslinked by 0.1 % bis-acrylamide; the lower reservoir buffer was 0.4244 M in Tris–HCl, pH 9.18. Two mg of ghost were dissolved in 1 ml of a solution containing 1 % sodium dodecylsulfate, 0.054 M Tris–H_2SO_4, 2 % sucrose and 0.1 % dithiothreitol; this was boiled for 3 min to complete solubilization. The electrophoresis was carried out at 3 mA per gel for 2.5 h and the gels then stained with Comassie blue. The mobility was measured from the top of the lower gel and the position of the front was shown by the position of the tracking dye (Bromophenol Blue). Approximate molecular weights were determined by the mobility of standard purified proteins, which are myosin heavy chain $212 \cdot 10^3$, β-galactosidase $130 \cdot 10^3$, bovine serum albumin $68 \cdot 10^3$, ovalbumin $46 \cdot 10^3$, cytochrome c dimer $26 \cdot 10^3$ and cytochrome c $13 \cdot 10^3$. The myosin (from bovine muscle) was generously supplied by Dr M. Yamaguchi, Muscle Biology Laboratory, University of Wisconsin. AMP-P(NH)P was obtained from ICN Isotope and Nuclear Division, Cleveland, Ohio. Calcium was determined by atomic absorption as described by Parker et al. [21]. One volume of a suspension of ghosts

(about 25 % ghosts by volume) was mixed with four volumes of a solution containing 0.2 M H_2SO_4 and 0.5 % Triton X-100 in Ca^{2+}-free water. Calcium was then determined using a Perkin-Elmer 403 Atomic Absorption Spectrophotometer with heated graphite atomizer.

RESULTS

As described by many investigators, the properties of isolated erythrocyte ghosts vary considerably depending on the method used for preparation and the species of animal from which the erythrocytes were taken [22]. Even when an established method was applied for isolation of ghosts from a single species, the properties still varied somewhat from one batch to another. Therefore, it is necessary to describe some typical properties of the erythrocyte membranes which were used in the experiments described here. Table I summarizes some properties of the freshly isolated porcine erythrocyte ghost membranes. The isolated ghosts were free from hemoglobin, which was demonstrated both by direct determination and by acrylamide gel electrophoresis. The phospholipid/protein ratio showed little variability, indicating that the amount of protein per ghost was also constant, since it has been shown that the amount of phospholipid per ghost is very stable [23, 24]. The sialic acid content of porcine erythrocyte ghosts was lower than that of human ghosts. When neuraminidase digestion was utilized for the assay, only 20 ± 5 nmoles of sialic acid were released per mg of ghost protein, while acid hydrolysis showed 34.5 ± 2.5 nmoles per mg of protein. In human cells, neuraminidase released nearly as much sialic acid

TABLE I

SOME PROPERTIES OF PORCINE ERYTHROCYTE GHOSTS

A number of batches were prepared by the standard method, and the parameters measured. The number of different batches observed is reported as *n* in the second column, and the standard deviations are given. The batch to batch variability was much greater than the error of the measurements, except for the hemoglobin content. All of the enzyme activities are reported as nmoles · min^{-1} · mg^{-1} at 37 °C, where mg refers to the amount of ghost protein. Sialic acid content is reported as nmoles · mg^{-1}. The salt concentrations utilized for the ATPase and *p*-nitrophenylphosphate assays were 3 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl and 0.05 mM $CaCl_2$.

ATPase		
Mg^{2+}	8.0±4.0	10
Na^+, K^+, Mg^{2+}	11.0±3.0	10
Ca^{2+}, Mg^{2+}	31.0±6.0	8
Na^+, K^+, Ca^{2+}, Mg^{2+}	38.0±6.0	4
p-Nitrophenylphosphate hydrolysing activity		
pH 5.0, Mg^{2+}	12.0±3.0	5
pH 5.0, K^+, Mg^{2+}	16.0±2.0	5
pH 7.0, Mg^{2+}	5.0±1.0	5
pH 7.0, K^+, Mg^{2+}	6.0±1.0	5
Acetylthiocholinesterase	60±10	4
Sialic acid	34.5±2.5	10
Hemoglobin/total protein	0.000 (w/w)	10
Phospholipid/protein	0.49±0.02 (w/w)	6

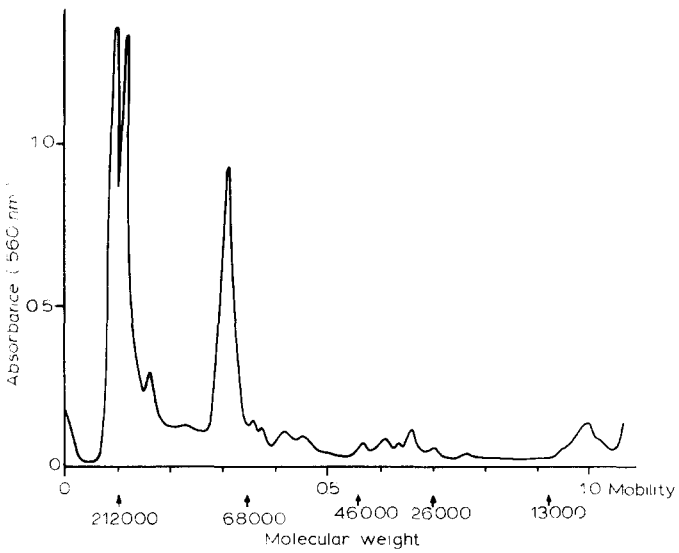

Fig. 1. Sodium dodecylsulfate–polyacrylamide gel electrophoresis pattern of isolated porcine erythrocyte ghosts. The band with a relative mobility of 0.32 was much narrower than the corresponding band from human erythrocyte ghosts. The molecular weights marked along the abscissa show the mobilities of the reference proteins.

as did acid hydrolysis [25].

ATPase activities were extremely variable even though the procedure for the isolation of ghosts was kept constant [26]. The results in Table I show the average values obtained from a number of different preparations. *p*-Nitrophenylphosphate hydrolysing activity was observed over a pH range of 4–9, and showed its highest activity at pH 5.0; at this pH, a ouabain sensitive potassium activation was observed. At pH 7.0, which was near the pH optimum for the *p*-nitrophenylphosphate hydrolysing activity from human ghosts [27, 28] the activity in porcine ghosts was much lower than at pH 5.0. The isolated ghosts showed the crenated shape [29] in 50 mM TES–triethanolamine buffer under phase contrast microscopy observation. Electron micrographs were also taken, giving results similar to those previously observed [1].

Endocytosis activity was checked in every preparation and gave 90–100 % activity. During the experiment, the crenated shape was observed at the beginning of the incubation in Mg–ATP, then the discoid shape appeared, and vacuoles began to be taken inside after 10–15 min.

The acrylamide gel electrophoresis pattern of the ghosts is shown in Fig. 1. Most of the components were similar to those of human erythrocytes as seen in our laboratory and reported by others [25, 30, 31]. Major peptides appeared with molecular weights of $220 \cdot 10^3$, $200 \cdot 10^3$ and $90 \cdot 10^3$.

Requirement for ATP hydrolysis in the process of endocytosis

It has been made clear that the presence of ATP is required to cause endocytosis. However, there still remain many questions about the role of the ATP molecule in the process of endocytosis. Although inhibitors of ATP synthesis such as sodium fluoride also inhibit endocytosis in whole erythrocytes [2], and ATPase

inhibitors showed the same effect on endocytosis [11], this did not demonstrate conclusively that ATP hydrolysis was required. The possibility could not be eliminated that the binding of ATP or ATPase products to the membrane somehow caused configurational changes of the membrane structure, resulting in endocytosis. In order to clarify this point, AMP-P(NH)P [32, 33], an ATP analogue in which the oxygen joining the γ and β phosphorus atoms were replaced by NH, was examined. This compound is resistant to enzymatic hydrolysis by ATPase. This was shown by the fact that inorganic phosphate was not detectable even after 60 min reaction of AMP-P(NH)P with ghosts.

If AMP-P(NH)P has the same affinity for the active site as does ATP, it should be a very effective competitive inhibitor of the ATPase. Kinetic analysis of ATP and AMP-P(NH)P with respect to ATPase has been carried out and the data are shown in Figs 2 and 3. The K_m of ATP for ATPase (when measured in the presence of Mg^{2+} only) was $2.0 \cdot 10^{-4}$ M. AMP-P(NH)P inhibited this ATPase competitively with a K_i of $2.0 \cdot 10^{-4}$ M. Thus AMP-P(NH)P was an extremely good analog of ATP in this system, and appeared to have an identical affinity for the active site, since the K_i for AMP-P(NH)P was the same as the K_m for ATP.

Thus, a good analog for ATP was available, and the effect of product or of reactant analog on endocytosis could be observed. In the absence of ATP, neither AMP-P(NH)P nor the combination of ADP and orthophosphate, nor ADP alone, caused endocytosis. The endocytosis assays were carried out at seven different concentrations ranging from 0.2 to 10 mM of each of these nucleotides. In the case in which ADP and orthophosphate were added together, each was present at these concentrations. In none of these experiments was any endocytosis observed. Clearly, simply binding of either ADP and orthophosphate, or of AMP-P(NH)P, did not cause endocytosis, but the hydrolysis of ATP was necessary. Since AMP-P(NH)P

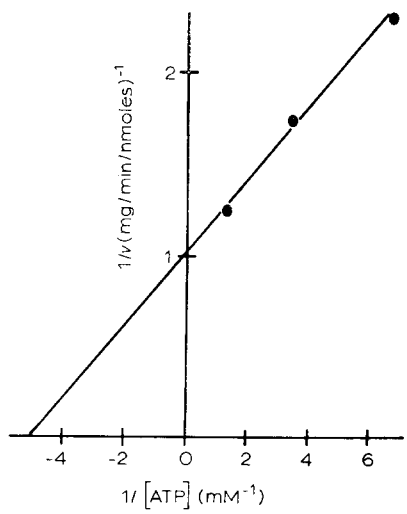

Fig. 2. K_m of ATPase activity for ATP. ATPase was measured in the presence of 3 mM Mg^{2+} as described in the Methods, except that the assay was carried out for only 10 min, in order to obtain the initial rate as nearly as possible. Points were obtained in triplicate and agreed with one another within 3 %.

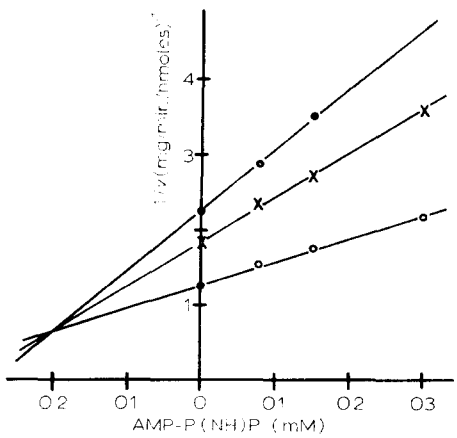

Fig. 3. Competitive inhibition of ATPase activity by AMP-P(NH)P. Dixon plot of three substrate concentrations; ATP concentrations were, filled circles, 0.15 mM; crosses, 0.30 mM; open circles 0.75 mM. Other conditions were the same as for Fig. 2.

acts as a competitive inhibitor for Mg–ATPase, it is to be expected that it would also inhibit competitively endocytosis supported by ATP splitting. It is not possible to quantitatively measure this competitive inhibition, due to the nature of the endocytosis assay, which measured the number of ghosts with vacuoles inside, rather than the amount of area taken into vacuoles. However, Fig. 4 clearly shows that AMP-P(NH)P inhibited endocytosis in a competitive manner. Since Mg^{2+} was present in considerable excess (3 mM, while 0.1 mM was sufficient for maximal ATPase), this inhibition was not due to chelation of Mg^{2+}. In accordance with the model of competitive inhibition, at higher ATP concentrations, higher concentrations of AMP-P(NH)P were required to inhibit endocytosis to the same degree.

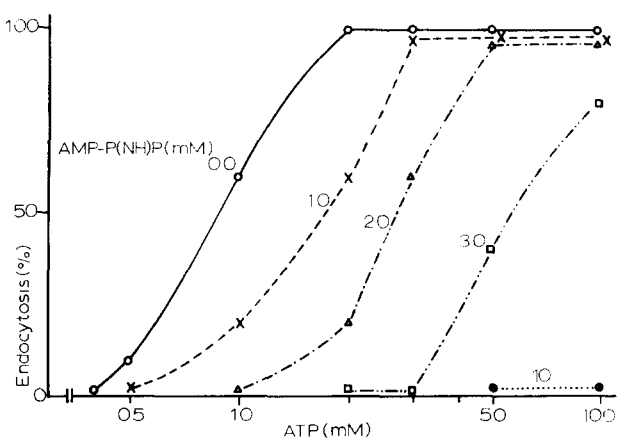

Fig. 4. Competitive inhibition of endocytosis by AMP-P(NH)P. Assays were carried out as described in Methods, except that the indicated concentrations of ATP and AMP-P(NH)P were added. Concentration curves were also run at 0.5 and 5 mM AMP-P(NH)P, yielding curves at intermediate positions.

Effects of Na^+, K^+ and Ca^{2+}

The requirement of Mg^{2+} for endocytosis has been made clear. The results shown in Table II confirm this point and show that neither EDTA nor Na^+ and K^+ stimulate endocytosis. The presence of sodium and potassium at the concentrations

TABLE II

ENDOCYTOSIS AND ATPase REQUIRE Mg^{2+}

The assay was carried out in 50 mM triethanolamine–TES buffer, except that the concentration of this buffer was lowered to 10 mM when Na^+ and K^+ were present. EDTA was present at a concentration of 1 mM, ouabain at 0.2 mM and other reagents as described in Methods. Endocytosis is expressed as the percent of ghosts containing internal vacuoles and the ATPase activity units and the ion concentrations are the same as in Table I.

Solute added	Endocytosis (%)	ATPase activity (nmoles · min^{-1} · mg^{-1})
None	0	<0.5
EDTA	0	<0.5
EDTA, Na^+, K^+	0	<0.5
EDTA, Mg^{2+}	0	<0.5
Mg^{2+}	100	10.0 ± 2.0
Mg^{2+}, Na^+, K^+	100	12.0 ± 2.0
Mg^{2+}, Na^+, K^+, ouabain	100	10.0 ± 2.0

at which they maximally activated the ATPase had no effect on endocytosis. Fig. 5 shows the effect of ouabain on ATPase and endocytosis. Ouabain showed no inhibitory nor activatory effect on endocytosis, while it inhibited the increment of ATPase activity caused by the addition of Na^+ and K^+. These results suggest that the Na^+, K^+ transport system is not related to the endocytosis system.

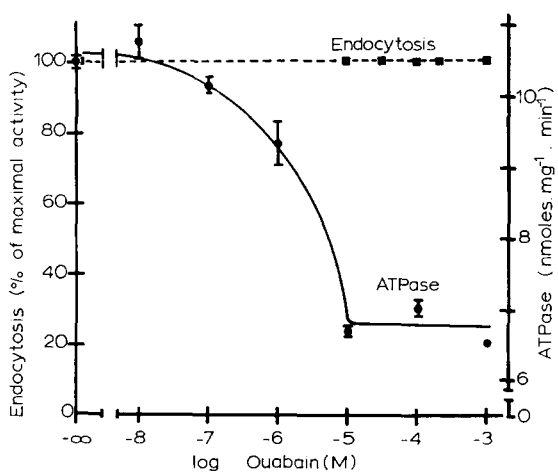

Fig. 5. Effect of added ouabain on endocytosis and ATPase activity in the presence of 3 mM Mg^{2+}, 100 mM Na^+ and 20 mM K^+.

In contrast to results reported for resealed ghosts [4], our preparation showed only an inhibitory effect of Ca^{2+} on endocytosis. This inhibition occurred at Ca^{2+} concentrations of 0.1 mM and above, and was accompanied by an increased ATPase activity in the presence or in the absence of Na^+ and K^+ (Figs 6 and 7).

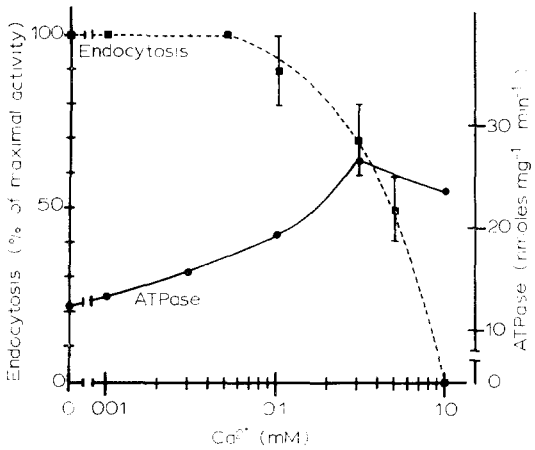

Fig. 6. Effect of Ca^{2+} on endocytosis and ATPase activity in the presence of 3 mM Mg^{2+}. Concentrations of Ca^{2+} which substantially stimulate ATPase also inhibit endocytosis.

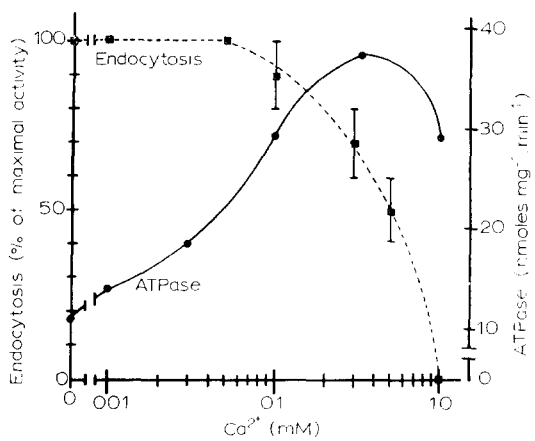

Fig. 7. Effect of Ca^{2+} on endocytosis and ATPase activity in the presence of 3 mM Mg^{2+}, 100 mM Na^+ and 20 mM K^+. Although stimulation of ATPase activity by Ca^{2+} was greater under these conditions, the effect of Ca^{2+} on endocytosis was unchanged.

Table III shows the effects of EGTA in preventing or reversing the action of Ca^{2+}. When EGTA was present with Ca^{2+}, the inhibitory effect of calcium was prevented by chelation. After a 30 min incubation in the presence of Ca^{2+}, Mg^{2+} and ATP (no EGTA), endocytosis did not occur. But further incubation of the same ghosts in the presence of ATP after addition of 1 mM EGTA did cause complete endocytosis, demonstrating the reversibility of the Ca^{2+} effect.

A subsequent investigation of ghosts which had been resealed by the method of Schrier et al. [4] yielded only small differences between ghosts resealed in the

TABLE III

REVERSIBILITY OF Ca^{2+} INHIBITION OF ENDOCYTOSIS

Solutes added[a]	Endocytosis (%)	ATPase nmoles · mg^{-1} · min^{-1}
1 mM Ca^{2+}	0	<1.0
0.5 mM Ca^{2+}, 3 mM Mg^{2+}	50	32.6
0.5 mM Ca^{2+}, 3 mM Mg^{2+}, 1 mM EGTA	100	4.4
1 mM EGTA, 3 mM Mg^{2+}	100	3.9
1 mM Ca^{2+}, 3 mM Mg^{2+}	0	28.5
1 mM EGTA, 3 mM Mg^{2+}[b]	100	10.2

[a] The basic medium contained only buffer, ghosts and ATP as described in Methods.

[b] After the initial incubation in the presence of Ca^{2+}, Mg^{2+} and ATP, the ghosts were centrifuged and the pellet resuspended in a medium containing Mg^{2+}, EGTA, ATP and buffer, and were incubated 30 min longer. The centrifugation was necessary to remove the phosphate released during the first 30 min, allowing the assay of ATPase during the second incubation.

presence and absence of 1 mM Ca^{2+}. Electron micrographs of ghosts incubated up to one h showed no significant differences in the amount of membrane incorporated into the two types of resealed ghosts. Ca^{2+} measurements showed that, though the cells were resealed in the presence of 1 mM Ca^{2+}, at the time when vesicles began to be observed in the preparation (10 min after the addition of inducer) the actual Ca^{2+} concentration was 30 nmoles of Ca^{2+}/mg of protein. In comparison ghosts resealed in the absence of Ca^{2+} showed a concentration of 20 nmoles Ca^{2+}/mg of protein at the same point in the incubation. Thus, the incorporation of Ca^{2+} resulted in only a 10 nmoles Ca^{2+}/mg of protein increase in the overall Ca^{2+} content.

DISCUSSION

Changes in erythrocyte shape between the disc form and the crenated form had been reported to be affected by the level of ATP in the cell and it was suggested that a contractile system existed in red cells [10]. Penniston and Green [1] discovered that isolated ghosts performed endocytosis (internalization of vacuoles) in the presence of ATP and suggested that hydrolysis of the high energy bond of the ATP molecule provided the energy to drive the configurational change of the membrane. ATP-dependent endocytosis in whole erythrocytes was also observed in the presence of primaquine [2, 6]. It is thus clear that erythrocyte membranes do perform endocytosis and that ATP is required for the process. The studies reported here have focused on the relationship between endocytosis and ATPase activity of the membrane and on the necessity of ATP hydrolysis for endocytosis. The data shown concerning the general ghost properties demonstrate the purity of this hemoglobin-free ghost preparation. Judging from the ATPase activity, the ghosts as prepared are quite permeable to ATP, as is expected for ghosts prepared in EDTA [24]. The sodium dodecylsulfate polyacrylamide gel electrophoresis pattern shows that the porcine erythrocyte membrane components are almost the same as those of human erythrocyte membranes, except that the band around $90 \cdot 10^3$ mol. wt is narrower and sharper than that of human.

The requirement for ATP hydrolysis in the muscular contraction process was clearly shown using AMP-P(NH)P by Yount et al. [32, 33]. They showed that AMP-P(NH)P was not hydrolysed by the myosin system but that it was the most potent competitive inhibitor of ATP, showing a K_i value very close to the K_m of the ATPase for ATP. This analog was a better competitive inhibitor for a variety of ATPase enzymes than was the related methylene bridge compound [33]. This ATP analog cannot be substituted for ATP to generate endocytosis nor can it be hydrolysed by the ATPase in erythrocyte ghosts. Other nucleoside phosphates, such as GTP, ITP, ADP or AMP cannot be substituted in this ghost preparation. These results clearly show not only that ATP must be hydrolysed to cause endocytosis but that the requirement for ATP is specific. It has been reported that ADP can be substituted for ATP in stimulating endocytosis in resealed erythrocyte ghosts [2]. The resealed ghosts retain much more hemoglobin, and presumably more glycolytic enzymes, than do the ghosts described here, so the effect of ADP in that system may be due to its phosphorylation by glycolysis.

The effect of Ca^{2+} on endocytosis is interesting and difficult to interpret. The results reported in this paper are different from those of Schrier et al. [4], which reported that added Ca^{2+} activated endocytosis at concentrations of 0.05–1.0 mM and then inhibited endocytosis at higher concentrations. This disagreement may be attributed to differences in the ghost preparations and in the methods of assay used for endocytosis. The resealed ghost preparation, used by Schrier et al., retains the impermeability of the intact red cell to ions [35]. The moderate amount of Ca^{2+} originally added to the resealed ghosts would be greatly reduced by the operation of the Ca^{2+} pump [36], which would lower the level of Ca^{2+} inside the ghost. Thus, if Ca^{2+} stimulated endocytosis, it must have been a very low Ca^{2+} concentration which did so. The method used for endocytosis assay by Schrier et al. was binding of ^{57}Co vitamin B_{12} to specific membrane sites. This binding requires Ca^{2+} and serum globulin: this requirement for Ca^{2+} may make the interpretation of the results more difficult. From the results of our experiments (Figs 6 and 7), it is clear that high levels of Ca^{2+} had an inhibitory effect, but no effect of low levels of Ca^{2+} was observed. The effect of low levels of Ca^{2+} is currently being investigated more extensively in our laboratory.

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