Vacuole Formation in Human Erythrocyte Ghosts[1] (37367)

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Study of human erythrocyte ghosts has been pursued as a means of exploring characteristics of an accessible plasma membrane. One of these functions is the phenomenon of endocytosis which was observed and described by Penniston and Green (1). They noted that when ATP was resealed within erythrocyte ghosts, endocytosis began and led to vacuole formation. More recently we described a method for quantifying vacuole formation and were, therefore, able to show that Mg^{2+} was required in addition to ATP and proposed that the actual substrate for endocytosis was Mg^{2+} ATP (2). When 2–20 m*M* Ca^{2+} was added, ghost vacuole formation was strongly inhibited. Therefore, there appeared to be an interaction of Ca^{2+}, ATP and Mg^{2+} involved in erythrocyte ghost vacuole formation. Several authors have hypothesized that there is an interaction of ATP, Mg^{2+} and Ca^{2+} at the inner surface of the erythrocyte membrane such that ATP antagonizes the action of Ca^{2+} (3) which tends to contract the erythrocyte membrane (4), and make it rigid (3). In some of these studies Mg^{2+} works similarly or in additive fashion with ATP antagonizing Ca^{2+} action, as in preserving deformability characteristics of erythrocyte membranes (3). Other observers measuring Ca^{2+} mediated ghost shrinkage report no Mg^{2+} effect (4). In deformability studies, ATP can be replaced by EDTA, but not by the nucleotide triphosphates GTP or ITP; therefore, Ca^{2+} chelation is important in preserving erythrocyte ghost deformability (3). Conversely, GTP and ITP are better substitutes for ATP than is EDTA in reversing Ca^{2+} mediated ghost shrinkage (4). These observations suggest that some sort of energization is more important than Ca^{2+} chelation in control of cell size. Ghost vacuole formation involves critical interaction of the three components Ca^{2+}, Mg^{2+} and ATP, and the object of this report was to explore the role of each in erythrocyte vacuole formation. The relative importance of Ca^{2+} chelation or membrane energization in vacuole formation was studied by substituting a variety of substrates as well as EDTA for ATP.

Materials and Methods. Substrates were obtained from the Sigma Chemical Company, Cal Biochem or P. L. Biochemical Company.

Methods for measuring quantitative vacuole formation were essentially those previously reported (2). Four important modifications in methodology were made. During initial exposure to hypotonic media 20 vol instead of 10 vol of hypotonic solution (30 mOsm/kg H_2O) were added to 1.0 ml of packed RBC. The larger volume produced more uniform incorporation and resealing of substrates along with less retention of the initial hemolysate. Where differing amounts of ATP, Mg^{2+} or Ca^{2+} were added during the hemolysis reversal stage, sufficient NaCl was supplied to maintain the osmolarity of hemolysis at 30 mOsm/kg. The restoration of isotonicity was achieved by addition of 1.54 *M* KCl which brought the osmolarity to 308 mOsm/kg. Where indicated, 2.5 m*M* $MgCl_2$ was added. Ca^{2+} was no longer (2) routinely included in the resealing step, because we had discovered that Ca^{2+} interfered with vacuole formation and that one reason concentrations of ATP in the order of 5 and 10 m*M* were required was to block the Ca^{2+} inhibition (2). Ghost particle counts were performed routinely in the Coulter Model B, so that all isotopic and substrate measurements could be related directly to the number of ghosts recovered. Lastly we had observed by the

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use of phase microscopy that vacuole formation had begun prior to incubation of the resealed and washed ghosts with the plasma containing vitamin B_{12} complexing agents and ^{57}Co vitamin B_{12}. Therefore substantial amounts of endocytosis and vacuole formation had occurred before the ^{57}Co vitamin B_{12} protein binding complex had been fixed to the exterior of the ghost plasma membrane. By the addition of 5 m*M* NaF during the resealing stage (2) it was possible to retard vacuole formation as visualized by phase microscopy, until the isotopic complex had been bound to the membrane. During the subsequent 30-min period of incubation at 37°, the NaF presumably diffused out of the ghost since vacuole formation was complete with almost all ghosts having one or more vacuoles. Incubation was carried out in one volume of plasma containing the plasma proteins complexed to ^{57}Co vitamin B_{12} and 1.5 volumes of Hanks' Solution, thereby maintaining osmolarity at 308 mOsm/kg. The concentrations of substrates, Ca^{2+} and Mg^{2+}, indicated were added during the hemolysis reversal and resealing stages. The ^{57}Co isotopic counts recorded in the tables are the increments in cpm/ml packed erythrocyte ghosts over a ghost preparation made without substrate addition. As noted in our earlier report (2) there is an absolute requirement for Ca^{2+} in the binding of the serum protein–^{57}Co vitamin B_{12} complex to the erythrocyte membrane. Therefore, when experiments requiring EDTA were performed, the isotopic method for quantifying vacuole formation could not be used (2) and we relied on cruder morphologic estimates made by phase microscopy. Preparations where vacuoles were seen in 1–10% of ghosts were graded 1+, vacuoles in 11–50% of ghosts were graded 2+, vacuoles in 50–75% of ghosts were graded 3+, and vacuoles in 75–100% of ghosts were graded 4+.

ATP was measured enzymatically by coupling the hexokinase and glucose-6-phosphate dehydrogenase reactions.

Results. Requirements of Ca^{2+}, Mg^{2+} and ATP for ghost vacuole formation. The data are summarized in Table I. Concentrations of 2.5 m*M* ATP during resealing produce optimum vacuole formation and increasing ATP has no further benefit (Table II). Mg^{2+} is required. The addition of Ca^{2+} has a biphasic effect. At concentration from 0 to 1.0 m*M*, it produces increased vacuole formation and at levels above 1.0 m*M*, Ca^{2+} retards vacuole formation.

TABLE I. ATP, Ca^{2+}, Mg^{2+} Requirements for Ghost Vacuole Formation.

Contents of resealing solution			Vacuole formation
ATP m*M*	Mg^{2+} m*M*	Ca^{2+} m*M*	Δ cpm/ml ghosts
2.5	0	0	0
2.5	2.5	0	240
2.5	2.5	0.1	243
2.5	2.5	0.25	524
2.5	2.5	0.5	769
2.5	0	0.5	123
2.5	2.5	1.0	1038
2.5	2.5	2.0	501
2.5	2.5	2.5	498
2.5	2.5	5.0	68
2.5	2.5	10.0	54

Substrate requirements for vacuole formation. The inability of EDTA to substitute for ATP in the presence and absence of Mg^{2+} is noted in Table II. Other substrates were tested for their ability to form vacuoles. Two conditions were chosen for purposes of comparison: 2.5 m*M* substrate and 2.5 m*M*

TABLE II. Ghost Vacuole Formations with Varying Concentrations of ATP and EDTA (Δ cpm/ml ghosts).

	Contents of resealing solution		
	No divalent cations	2.5 m*M* Mg^{2+}	2.5 m*M* Mg^{2+} and 1.0 m*M* Ca^{2+}
2.5 m*M* ATP	0	520	1049
5.0 m*M* ATP	0	339	970
10.0 m*M* ATP	0	410	471
0.5 m*M* EDTA	0[a]	0[a]	
2.5 m*M* EDTA	0[a]	0[a]	
5.0 m*M* EDTA	0[a]	0[a]	

[a] Based on morphologic evaluation by phase microscopy. For purposes of comparison, 2.5 m*M* ATP and 2.5 m*M* Mg^{2+} was graded 3+, while 2.5 m*M* ATP and 2.5 m*M* Mg^{2+} and 1 m*M* Ca^{2+} was graded 4+.

TABLE III. Substrate Requirements for Vacuole Formation (Δ cpm/ml ghosts).

	2.5 m*M* Mg^{2+}		2.5 m*M* Mg^{2+} and 0.5 m*M* Ca^{2+}	
		% ATP value		% ATP value
ATP	475	100	750	100
Triphosphates				
GTP	115	29	190	25
UTP	145	31	175	23
CTP	80	17	85	11
ITP	190	40	325	43
Diphosphates				
ADP	440	93	570	76
GDP	120	25	255	34
UDP	130	27	155	21
Monophosphates				
AMP	85	18	45	6
cAMP	85	18	90	12
GMP	0	0	0	0
UMP	15	3	20	3

$MgCl_2$ without and with addition of 0.5 m*M* Ca^{2+} during resealing (Table III). Using 5.0 m*M* substrate concentration gave the same results. Surprisingly, ADP was almost as effective as ATP. In general, the nucleotide monophosphates were less effective than the nucleotide di- and triphosphates. No clear distinction between purine and pyrimidine bases appeared.

Discussion. In the formation of erythrocyte vacuoles there is a strict requirement for Mg^{2+}, in the absence of which almost no vacuoles form. A substrate is also required and ATP seems to function best. It should be understood that the hemolysis reversal technique used here does not provide "white ghosts" devoid of hemoglobin and substrates, there being about 5–10% of the original hemoglobin retained along with glycolytic capacities (5). ADP was quite effective as a substitute for ATP, while other nucleotides were generally less effective. Perhaps ADP is converted to ATP via erythrocyte adenylate kinase (6). Chelation of Ca^{2+} by EDTA did not reproduce ATP results. The effect of Ca^{2+} was surprisingly biphasic, small amounts potentiating vacuole formation and large amounts inhibiting vacuole formation. Ca^{2+} mediated inhibition was anticipated since Ca^{2+} resealing into ghosts results in remarkable increase in rigidity and constriction of ghost size perhaps by activation of a contractible actomyosin. These changes would mechanistically interfere with vacuole formation. The potentiating effect of low concentrations of Ca^{2+} is not easily understood. Small amounts of Ca^{2+} are known to activate a Ca^{2+}, Mg^{2+} ATPase (7) which may have a role in endocytosis and vacuole formation.

Summary. Human erythrocyte ghost endocytic vacuole formation requires Mg^{2+} and ATP and occurs optimally in the presence of 0.5–1.0 m*M* Ca^{2+} with larger amounts of Ca^{2+} producing inhibition of vacuole formation. ADP can substitute for ATP as substrate but other nucleotides are much poorer substitutes. EDTA cannot produce ghost endocytic vacuoles. Therefore, in ghost vacuole formation, Ca^{2+} chelation seems less important and relatively specific energization by ATP or ADP is more important.

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