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Interaction Between Cytoplasmic (Ca^{2+}—Mg^{2+}) ATPase Activator and the Erythrocyte Membrane

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Human red blood cells (RBC) contain a cytoplasmic, nonhemoglobin protein which activates the (Ca^{2+}-Mg^{2+})ATPase of isolated RBC membranes. Results presented in this paper confirm that activation of (Ca^{2+}-Mg^{2+})ATPase is associated with binding of the cytoplasmic activator to the membrane. Binding of the cytoplasmic activator is reversible and dependent on ionic strength and Ca^{2+}. Cytoplasmic activator is sensitive to trypsin but is not degraded when intact RBC are exposed to trypsin. Cytoplasmic activator does not modify the (Ca^{2+}-Mg^{2+})-ATPase of membranes from RBC exposed to activator prior to hemolysis. Thus, the activator is located in the cell and appears to act by binding to the inner membrane surface.

Key words: cytoplasmic activator, red blood cells, membrane ATPase, Ca^{2+} transport, (Ca^{2+}-Mg^{2+})ATPase

Several years ago, Bond and Clough (1) reported on a curious kind of activity which was found in the membrane-free hemolysate of the human red blood cell (RBC). The activity, which was due to a nonhemoglobin protein, increased the (Ca^{2+}-Mg^{2+})ATPase but not the Mg^{2+}-ATPase or (Na^{+}-K^{+}-Mg^{2+})ATPase activity of isolated RBC membranes. The material will be referred to as "cytoplasmic activator" or "activator" in this communication.

(Ca^{2+}-Mg^{2+})ATPase of RBC membranes has been associated with active transport of Ca^{2+} (2–4). On the other hand, some workers have also suggested that RBC (Ca^{2+}-Mg^{2+})-ATPase may be associated with a system of actomyosin-like fibers which maintain the flexibility and normal biconcave shape of the RBC (5–7). Thus, Bond and Clough (1) sug-

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gested that the cytoplasmic activator could be related to either Ca^{2+} transport, or cell flexibility and shape, or both. Luthra and co-workers partially purified the cytoplasmic activator (8) and demonstrated its existence in the RBC of newborn and adult animals of various species (9). They found it "provocative to consider that this activator may be related to Ca^{2+} transport in erythrocytes which is associated with (Ca^{2+}-Mg^{2+})ATPase or may be a part of a system of actomyosin-like fibers thought to maintain the flexibility and characteristic biconcave shape of the cell" (8).

We were led to investigate the cytoplasmic activator because of widely different (Ca^{2+}-Mg^{2+})ATPase activity of membranes from RBC hemolyzed in various buffers. The explanation is that the cytoplasmic activator can bind to the membrane in high ionic strength. Once binding occurs it is not reversed, even with repeated washings in low ionic strength buffer (10). We demonstrated that the cytoplasmic activator is present in excess in the RBC, that it binds physically to the membrane, and that binding is reversible and dependent on Ca^{2+} (10, 11). Evidence in this paper provides further data compatible with the idea that the cytoplasmic activator is a regulatory protein.

METHODS

Outdated packed human RBC were obtained from the local blood bank. All preparation steps were performed at 0–5°C. The packed cells were washed 3 times with 155 mM NaCl, removing any remaining buffy coat at each step. RBC were hemolyzed in 1 of 2 buffers (pH 7.4): 20 ideal milliosmolar (imosM) imidazole (I20), or 310 imosM imidazole (I310). Calculation of osmolarity was done using the Henderson-Hasselbalch equation assuming a pK_a value for imidazole of 7.05 (an Advanced osmometer gave a measured osmolality of 290 mOs/kg for I310). Hemolysis buffer was added rapidly and vigorously to a measured volume of packed, washed RBC with a buffer:cells ratio of 14:1. The resulting hemolysate was mixed thoroughly and centrifuged at 48,000 × g for 20 min. The pellet of packed ghosts was washed 4 times with I20 (pH 7.4) whether hemolyzed in I20 or I310. After each wash and centrifugation, the supernatant fluid was aspirated, and the tube was rotated to allow the loosely packed membranes to slide off the small pellet of unlysed cells and/or debris; the pellet was then aspirated. Membranes were washed once with 40 mM histidine-40 mM imidazole buffer, pH 7.1 (HI-40). An equal volume of HI-40 buffer was then added to the membranes, and the resulting suspension was stored in the refrigerator on ice. Protein content of the membrane suspension was determined by the method of Lowry et al. (12) using bovine serum albumin as the standard.

The ATPase incubation medium contained (in a final volume of 1 or 2 ml) ~100 μg membrane protein, 3 mM ATP (Na_2 ATP, neutralized to pH 7.1), 18 mM histidine-18 mM imidazole buffer (pH 7.1), 3 mM $MgCl_2$, 80 mM NaCl, 15 mM KCl, and 0.1 mM ouabain. $CaCl_2$ (0.1 mM) was added to appropriate tubes for determination of (Ca^{2+}-Mg^{2+})ATPase, which was taken as "extra" ATP splitting induced by Ca^{2+} addition in the presence of Mg^{2+} and ouabain. Tubes without Ca^{2+} gave Mg^{2+}-ATPase activity and also served as a blank to correct for nonenzymatic breakdown of ATP and for the presence of inorganic phosphate in the membrane preparation. All assay tubes (in duplicate) were incubated at 37°C in a shaking water bath for 1 h. The reaction was started by addition of substrate and stopped by the addition of one-half the incubation medium volume of ice-cold 1.5 N perchloric acid. After thorough mixing and centrifugation, 0.5 or 1 ml of supernatant was analyzed for inorganic phosphate using the method of Fiske and SubbaRow (13). Results are expressed as μmoles of inorganic phosphate (P_i) released·mg membrane protein^{-1}·h^{-1}.

TABLE I. RBC Membrane (Ca^{2+}-Mg^{2+})ATPase Activity: Influence of Hemolysis Buffer*

Hemolysis buffer	n	(Ca^{2+}-Mg^{2+})ATPase activity
I310	87	3.25 ± 0.07[a]
I20	46	0.76 ± 0.04
TP310	3	0.03 ± 0.02
U310	3	0.37 ± 0.01

*RBC were hemolyzed in various buffers and membranes were prepared and assayed according to Methods. Activities are expressed as μmole PO_4^{3-}·mg protein $^{-1}$·h^{-1}, mean ± SEM.
[a]Significantly greater than I20 membrane value, $P < 0.01$.

Endogenous (Ca^{2+}-Mg^{2+})ATPase activator was partially purified by the method of Luthra et al. (8). The sole modification of that method was in the use of imidazole buffers for hemolysis. In experiments in which activator was added to the incubation medium (0.2 ml activator fraction/1ml incubation medium), an equal amount of 20 mM Tris-maleate buffer, pH 6.8 at 25°C, was added to the control.

In all assays, conditions were such that less than 15% of total substrate was utilized during any incubation period. Enzymatic activity was linear with time and proportional to the amount of membrane protein added. Membranes were usually assayed on the day after preparation. Data were analyzed for statistical significance by Student's paired or unpaired t test as appropriate.

RESULTS

We recently found that the cytoplasmic activator of RBC membrane (Ca^{2+}-Mg^{2+})-ATPase (or simply cytoplasmic activator) can bind to the RBC membrane and that binding correlates with high (Ca^{2+}-Mg^{2+})ATPase activity (10, 11). Binding can occur during hemolysis under certain conditions. Thus hemolysis of RBC in isoosmotic (310 imosM) imidazole buffer (I310) results in membranes with very high (Ca^{2+}-Mg^{2+})ATPase activity (Table I). "I310 membranes" have bound cytoplasmic activator whereas "I20 membranes" (prepared from RBC hemolyzed in 20 imosM imidazole) have no associated cytoplasmic activator and have low (Ca^{2+}-Mg^{2+})ATPase activity (Table I). A question which arose as to whether an isoosmotic environment (such as that provided by I310) is sufficient to promote binding of cytoplasmic activator to the RBC membrane during hemolysis. RBC were hemolyzed in isoosmotic urea (U310) or 2,4,6-trimethylpyridine (TP310) solutions, pH 7.4. Membranes derived from these hemolysates did not have an enhanced (Ca^{2+}-Mg^{2+})ATPase activity compared to I20 membranes (Table I). Thus, an isoosmotic environment at the time of hemolysis is not sufficient to promote binding of cytoplasmic activator to the RBC membrane. The results in Table I might be interpreted to mean that imidazole promotes cytoplasmic activator binding in a somewhat specific fashion. However, ionic strength per se appears more important for binding of activator than does the presence of a high concentration of imidazole. This can be demonstrated by a somewhat different approach to hemolysis. RBC in 0.9% NaCl were freeze-thawed 3 times using dry ice/$CHCl_3$ –37°C H_2O bath. The broken RBC were then washed with I20 or I310 and membranes were prepared. As shown in Table II, there was no difference in activity between these treatments and each of these "freeze-thaw" membrane preparations had much higher (Ca^{2+}-Mg^{2+})ATPase activity than regular I20 membranes prepared from the same cells. Thus, the presence of 0.9% NaCl during lysis caused by freeze-thawing was apparently sufficient to promote significant activator binding.

TABLE II. RBC Membrane (Ca^{2+}-Mg^{2+})ATPase Activity: Influence of Freeze-Thawing*

Type of membrane	Pretreatment of intact cells	(Ca^{2+}-Mg^{2+})ATPase activity
I310	None	3.08
I20	None	0.87
I20	1 ml packed RBC in 14 ml 0.9% NaCl, freeze-thaw 3x in dry ice/$CHCl_3$ and 37°C H_2O	2.55
I310	1 ml RBC in 14 ml 0.9% NaCl, freeze-thaw 3x in dry ice/$CHCl_3$ and 37°C H_2O	2.21

*RBC were freeze-thawed, or not, and membranes were prepared and assayed according to Methods. Activities expressed as μmole PO_4^{3-}·mg protein $^{-1}$·h^{-1}.

TABLE III. RBC Membrane (Ca^{2+}-Mg^{2+})ATPase Activity: Influence of Cytoplasmic Activator Pretreatment of RBC*

Type of membrane	(Ca^{2+}-Mg^{2+})ATPase activity
I20	0.65
I20, from RBC treated with activator in 0.62% NaCl	0.68
I20 (plus added activator in incubation)	2.39
I20, from RBC treated with activator in 0.62% NaCl (plus activator added in incubation)	2.78

*Cytoplasmic activator was partially purified according to Luthra et al. (8). Intact RBC were treated, or not, with cytoplasmic activator in 0.62% NaCl. I20 membranes were then prepared and assayed according to Methods. ATPase activity is expressed as μmole PO_4^{3-}·mg protein $^{-1}$·h^{-1}.

Binding of cytoplasmic activator to membranes was shown to be reversible and, under appropriate conditions, to be dependent on Ca^{2+}. The presence of small amounts of Ca^{2+} promotes cytoplasmic activator binding. Chelation of Ca^{2+} with EGTA causes removal of cytoplasmic activator from membranes (11). We considered that reversible Ca^{2+}-dependent binding of cytoplasmic activator might represent a means by which intracellular Ca^{2+} could regulate the Ca^{2+} pump. Of course, in considering that the cytoplasmic activator might be a regulator of the plasma membrane Ca^{2+} pump, it seemed reasonable to predict that it acts on the inner membrane surface. However, neither our results nor those of other workers ruled out that the activator might bind to the outer membrane surface of broken RBC membranes to cause increased (Ca^{2+}-Mg^{2+})ATPase activity. The following experiment suggests that activator does not act at the outer membrane surface: Exposure of RBC to partially purified activator in 0.62% NaCl before preparation of I20 membranes neither increases (Ca^{2+}-Mg^{2+})ATPase activity nor does it prevent the (Ca^{2+}-Mg^{2+})ATPase activity of the resultant I20 membranes from being increased by activator during incubation (1) (Table III). The concentration of NaCl (0.62%) employed is sufficient both to allow the ionic-strength dependent interaction between activator and membrane and to prevent hemolysis. Since there appears to be no significant change in (Ca^{2+}-Mg^{2+})ATPase activity from this pretreatment, these data suggest that the activator does not interact with the outside of the membrane and that access to the cytoplasmic surface must be achieved.

TABLE IV. RBC (Ca^{2+}-Mg^{2+})ATPase Activity: Influence of Pretreating RBC With Trypsin or Trypsin Plus Trypsin Inhibitor*

Type of membrane	n	(Ca^{2+}-Mg^{2+})ATPase activity
I310	2	2.17 ± 0.03
I20	2	1.03 ± 0.06
I20, RBC pretreated with trypsin	2	0.23 ± 0.07[a]
I20, RBC pretreated with trypsin plus trypsin inhibitor	2	1.30 ± 0.12

*RBC were pretreated with trypsin or trypsin plus trypsin inhibitor as noted in the text. Membranes were then prepared and assayed as in Methods. ATPase activity is expressed as μmole PO_4^{3-}·mg $protein^{-1}$, mean ±SEM.

[a]Significantly less than I20 membrane value.

The question arose as to whether cytoplasmic activator is really cytoplasmic. Since hemolysis is necessary to detect cytoplasmic activator binding, it is possible that the activator is an outer surface protein and is released from the RBC surface upon hemolysis. It could then gain access to the inner surface (and to the hemolysate). To rule this out, we carried out experiments on partially purified activator and on intact RBC using trypsin and trypsin inhibitor. Activity of partially purified activator was completely lost upon incubation with trypsin (0.5 mg/ml) for 2 h at 25°C. RBC were incubated at 25°C in trypsin, 0.5 mg/ml, in 0.9% NaCl for 2 h, after which trypsin inhibitor, 1 mg/ml, was added. No hemolysis occurred during this procedure or in the control preparation of RBC incubated with trypsin plus trypsin inhibitor; 0.5 and 1.0 mg/ml, respectively. Both groups of RBC were washed twice in 0.9% NaCl and then I20 membranes were prepared. The respective hemolysates were collected and the activator was isolated by the method of Luthra et al (8). Results demonstrate that treatment of intact RBC with trypsin did not decrease activator effectiveness of the subsequent hemolysate (no difference from trypsin plus trypsin inhibitor control). Determination of the (Ca^{2+}-Mg^{2+})ATPase activity of the respective I20 membranes derived from these RBC showed that trypsin pretreatment significantly decreased (Ca^{2+}-Mg^{2+})ATPase activity of I20 membranes (Table IV). RBC pretreatment with trypsin plus trypsin inhibitor caused a small, nonsignificant ($P > 0.05$) increase in (Ca^{2+}-Mg^{2+})ATPase activity. These data suggest that the (Ca^{2+}-Mg^{2+})ATPase, but not the activator, is accessible to trypsin in intact RBC.

DISCUSSION

Results of this and related studies demonstrate the existence in human RBC of a cytoplasmic activator of (Ca^{2+}-Mg^{2+})ATPase. Effectiveness of the activator is associated with its binding to the RBC membrane, apparently at the inner membrane surface. Treatment of intact RBC with activator results in no effect, and trypsin treatment in intact RBC results in no loss of activator. Thus, the activator appears to be located in the cell and is almost certainly a cytoplasmic protein since it is so readily soluble (1, 8, 10).

Because at least some of the (Ca^{2+}-Mg^{2+})ATPase of the RBC membrane is associated with Ca^{2+} transport (2–4), it is suggested that the cytoplasmic activator is a regulator of the Ca^{2+} pump. Support for this suggestion can be found in the recent work of Macintyre and Green (14) who added membrane-free RBC hemolysate to inside-out RBC membrane vesicles. The uptake of $^{45}Ca^{2+}$ into the inside-out vesicles was increased by 50% under

their conditions. It must be admitted that there may be more than one (Ca^{2+}-Mg^{2+})ATPase in the RBC membrane (15–19). Thus, it is possible that some additional, or other, function of the RBC membrane is influenced by cytoplasmic activator. We prefer to think of the cytoplasmic activator as an "activator" of the Ca^{2+} pump but recognize that substantiation of this view will require further work.

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