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ARTICLES

Association of Vanadate-Sensitive Mg^{2+}-ATPase and Shape Change in Intact Red Blood Cells

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Abstract Intact human erythrocytes, initially depleted of Mg^{2+} by EDTA incubation in the presence of A23187, exhibit Mg^{2+}-dependent phosphate production of around 1.5 mmol per liter cells · h, half-maximally activated at around 0.4 mM added free Mg^{2+}. This appears to correspond to Mg^{2+}-stimulated adenosine triphosphatase (Mg^{2+}-ATPase) activity found in isolated membranes, which is known to have a similar activity and affinity for Mg^{2+}. Vanadate (up to 100 μM) inhibited Mg^{2+}-dependent phosphate production and ATP breakdown in intact cells. Over a similar concentration range vanadate (3–100 μM) transformed intact cells from normal discocytes to echinocytes within 4–8 h at 37°C, and more rapidly in Mg^{2+}-depleted cells. The rate of Ca^{2+}-induced echinocytosis was also enhanced in Mg^{2+}-depleted cells. These results support previous studies in erythrocyte ghosts suggesting that vanadate-induced shape change is associated with inhibition of Mg^{2+}-ATPase activity localized in the plasma membrane of the red blood cell.

Key words: erythrocytes, magnesium, echinocyte, calcium, plasma membrane

Human red blood cells undergo both ATP-dependent and ATP-independent transitions from smooth biconcave (discocytic) forms to crenated (echinocytic) states [1]. Despite a considerable amount of detailed study over a number of years, the exact mechanisms of red blood cell shape regulation remain uncertain. The bilayer-couple hypothesis [2,3] proposes that cell shape is a function of the relative areas of the inner and outer leaflets of the membrane, such that anionic amphipaths, which accumulate mainly in the outer leaflet, cause echinocytosis by expanding the outer leaflet, whereas cationic amphipaths, which accumulate in the inner leaflet, produce cup shapes (stomatocytosis) by increasing the inner leaflet area. This hypothesis could also account for the ATP-dependent control of red cell shape [4], since lipid phosphorylation by ATP could expand the inner leaflet by increasing its mass and negative charge density [5]. Dephosphorylation of membrane inositol phospholipids during ATP-depletion may have the opposite effect and cause echinocytosis [6]. It has also been proposed that phosphorylation might be required for expansion of the cytoskeletal spectrin-actin network at the inner membrane surface [7], but echinocyte formation was subsequently shown to precede spectrin dephosphorylation [8]. Furthermore, ATP-dependent phosphorylation of spectrin could be dissociated from ATP-dependent shape change, since smoothing of echinocytes in red cell ghosts depleted of spectrin kinase was unaltered and both spectrin and phosphoinositide phosphorylation were unaffected by vanadate at concentrations which blocked shape changes in ghosts [9]. Echinocyte formation by ATP-depletion or Ca^{2+} loading of red blood cells has also been correlated with phosphoinositide [6] or phosphatidic acid [10] breakdown, resulting in shrinkage of the inner leaflet [6].

Other models of shape change have also been proposed. ATP-dependent translocation of aminophospholipids from the outer to the inner leaflet by the phospholipid translocase may serve to maintain the balance between the area of the bilayer halves [11,12]. In another proposal a direct effect of ATP binding to the cytoskeleton was postulated from results showing that ATP

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produced rapid shape changes in lipid-depleted cytoskeleton shells at low temperatures [13,14].

Vanadate has been used as a tool to unravel shape change mechanisms. Short exposure to vanadate inhibits drug-induced stomatocyte formation in intact red blood cells [15], but vanadate itself is an echinocytic agent after longer incubation times [16]. Vanadate inhibition of MgATP-dependent smoothing of echinocytic red cell ghosts was shown to correlate with inhibition of a fraction of Mg^{2+}-ATPase activity of these ghosts [15,17], but not with the other lipid or protein phosphorylation reactions examined [9].

In the present study we have characterized the Mg^{2+}-dependent ATP hydrolysis in intact red blood cells and investigated whether or not echinocyte formation induced by vanadate correlated with the inhibition of this enzymatic activity. We show that intact red blood cells have a Mg^{2+}-dependent ATP hydrolytic activity which is inhibited by vanadate at similar concentrations to those which promote echinocyte formation in these cells.

METHODS

A23187, EGTA, bovine serum albumin, inosine, iodoacetic acid, and sodium tetrathionate were obtained from Sigma Chemical Co. Dithiothreitol was from Boehringer Mannheim and ammonium metavanadate was ANALAR grade from BDH. All salts were analytical reagent grade. EDTA and trichloroacetic acid were from Ajax Chemicals.

Assay of Mg^{2+}-Dependent Phosphate Production in Mg^{2+}-Depleted Intact Red Blood Cells

To deplete intracellular Mg^{2+}, washed red blood cells (10% hematocrit) were preincubated in 60 mM NaCl, 75 mM KCl, 0.1 mM ouabain in 10 mM HEPES-sodium, pH 7.4, containing EDTA (4 mM) for 15 min at 37°C in the presence of 5 μM A23187, as previously described [18]. They were subsequently washed and incubated at 10% hematocrit in 60 mM NaCl, 75 mM KCl, 0.1 mM ouabain, 10 mM HEPES-sodium, pH 7.4, and 0.1 mM EGTA (Buffer A) and the reaction started by the addition of 20 μM A23187 and various concentrations of $MgCl_2$ for 60 min at 37°C with stirring. Cells were then lysed in trichloroacetic acid and the inorganic phosphate in the supernatant fraction was assayed as previously described [18,19].

Effect of Vanadate on Phosphate Production in Intact Red Blood Cells

Washed red blood cells (10% hematocrit) were suspended in Buffer A containing in addition 1 mM EGTA, 2 mM sodium tetrathionate, and various concentrations of ammonium metavanadate. Inorganic phosphate formation was measured following the incubation of cells at 37°C for 60 min, as described above. To decrease the basal inorganic phosphate content, red blood cells were first preincubated with 75 mM NaCl, 75 mM KCl, 10 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$, and 10 mM inosine for 30 min at 37°C and were washed five times before they were assayed [20].

Effect of Vanadate on ATP Hydrolysis in Intact Red Blood Cells

Washed red blood cells were suspended (10% hematocrit) in Buffer A containing in addition 1 mM iodoacetic acid and 2 mM sodium tetrathionate. After a 5-min preincubation at 37°C [39] cells were incubated with constant gentle stirring at 37°C for various time periods up to 2 h in the presence or absence of ammonium metavanadate (30 or 100 μM). Mg^{2+}-dependent hydrolysis of ATP was estimated by measuring ATP levels by HPLC using the method of Szabados and Christopherson [40]. To allow rapid quantitation of ATP isocratic elution with 250 mM KH_2PO_4, 500 mM KCl, pH 3.8, was used instead of the gradient elution described in their method and the ATP peak detected at 254 nm at a retention time of 7.5 min.

Influence of Vanadate on the Shape of Red Blood Cells

Normal or Mg^{2+}-depleted red blood cells were washed and incubated in Buffer A with gentle stirring at 37°C for various times in the presence or absence of various concentrations of ammonium metavanadate. To study the effect of Ca^{2+}, washed red cells were preincubated in the same conditions with 20 μM A23187 and various concentrations of $CaCl_2$. The percentage of red cells undergoing discocyte to echinocyte transition was estimated by phase contrast light microscopy, as described by Ferrel and Huestis [6] and Backman [16]. The transformation was also confirmed by scanning electron microscopy (results not shown).

RESULTS

Magnesium Dependence of Phosphate Formation in Intact Red Cells

Intact cells depleted of intracellular Mg^{2+} by incubation with EDTA in the presence of ionophore A23187 (see Methods) have a basal level of phosphate production of about 0.6 mmol/liter cells · h. Introduction of Mg^{2+} in the presence of A23187 progressively stimulated Pi production to a maximum of around 1.5 mmol/liter cells · h above basal levels at 1 mM $MgCl_2$ and above, with half-maximal activation at about 0.4 mM Mg^{2+} (Fig. 1).

Effect of Vanadate on Shape Change in Intact Red Blood Cells

The effect of vanadate on the shape transformation of red cells from discocytes to echinocytes was examined (Fig. 2). No echinocytes were formed within 8 h when control erythrocytes were incubated at 37°C, but when cells were incubated with vanadate from 3 to 100 μM, they were progressively transformed to echinocytes, the echinocytosis being complete after 4 h at 100 μM vanadate (Fig. 2).

Effect of Vanadate on ATP Breakdown in Intact Red Blood Cells

Attempts were made to determine the effect of vanadate on Mg^{2+}-dependent inorganic phosphate production in intact red blood cells. Incubation of cells with 40 μM vanadate, which

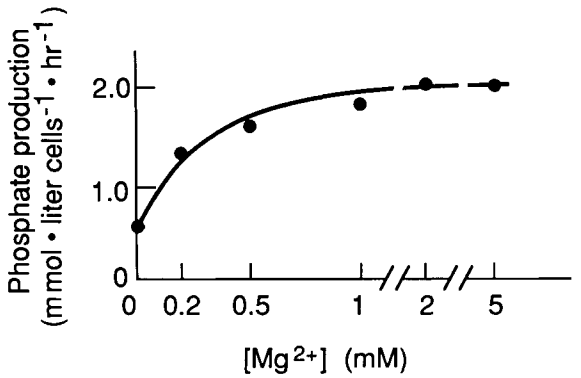

Fig. 1. Mg^{2+}-dependence of inorganic phosphate production in Mg^{2+}-depleted intact red blood cells. Mg^{2+}-depleted red cells (see Methods) were suspended in Buffer A containing various concentrations of Mg^{2+}. The ATPase reaction was started by the addition of 20 μM A23187 and the incubation continued for 60 min at 37°C. The activity before reintroduction of Mg^{2+} (about 0.6 mmol/liter cells · h) represents the basal intracellular phosphate production.

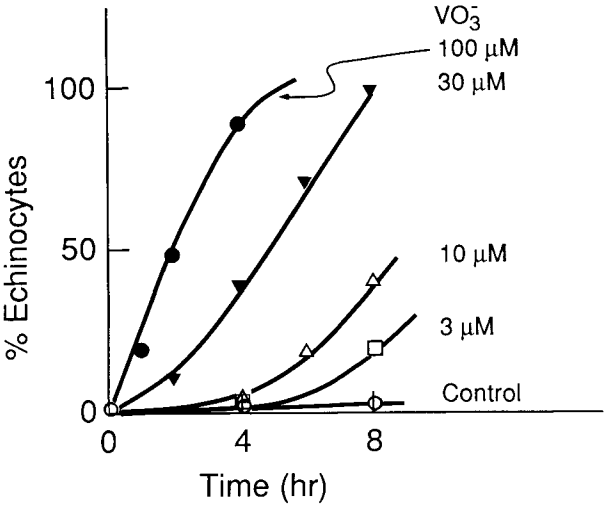

Fig. 2. Influence of vanadate on echinocyte formation in intact red blood cells. Washed intact red blood cells were incubated for various times at 37°C with increasing concentrations of vanadate: zero (0), 3 μM (□), 10 μM (△), 30 μM (▼), and 100 μM (●). The percentage of echinocyte formation was determined as described in Methods. The results are typical of 2 such experiments.

enters the cell through the anion channel [21,22], led to an increase in inorganic phosphate production (results not shown). Vanadate has been shown to increase phosphate production via 2,3-bisphosphoglycerate breakdown by the activation of the phosphatase activity of 2,3-bisphosphoglycerate synthase [23]. Consistent with the operation of this mechanism, 2 mM sodium tetrathionate (which blocks the breakdown of 2,3-bisphosphoglycerate [24]) eliminated this stimulating effect of vanadate at concentrations up to 100 μM (results not shown). However, vanadate still did not inhibit phosphate production in intact cells under these conditions (results not shown). When red blood cells were incubated with inosine (10 mM) for 30 min at 37°C, which decreased inorganic phosphate from 2.5 mM to 0.8 mM and is known to elevate ATP levels [20], vanadate inhibited phosphate production at concentrations up to 100 μM (Fig. 2). However, vanadate stimulated phosphate production at concentrations above 100 μM (Fig. 3).

The effect of vanadate on ATP hydrolysis was investigated more directly by measuring its effect on ATP levels over a two-hour incubation period. Vanadate progressively decreased ATP breakdown as a function of both concentration (30 and 100 μM) and time (1 and 2 h) (Fig. 4). This inhibition correlated closely with the formation of echinocytes, as determined from the experiments in Figure 2.

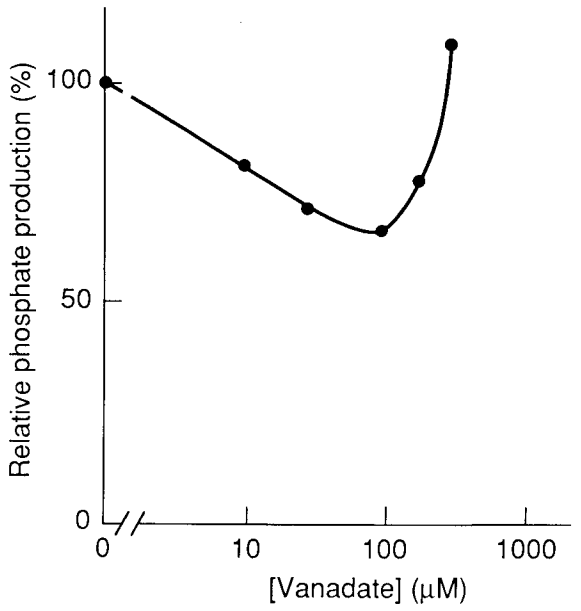

Fig. 3. Effect of vanadate on inorganic phosphate production in inosine-incubated intact red blood cells. Washed red blood cells were pre-incubated for 30 min in 75 mM NaCl, 75 mM KCl, 10 mM Tris (pH 7.4), 1 mM $MgCl_2$, and 10 mM inosine prior to assaying the inorganic phosphate released after 60 min incubation at 37°C in the presence of 2 mM sodium tetrathionate and various concentrations of vanadate (see Methods). The points are the average of 3 experiments. The activity in the absence of vanadate was 0.6 mmol/liter cells · h.

Effect of Magnesium and Calcium on Shape Changes in Intact Red Cells

Mg^{2+}-depletion also induced echinocyte formation, which was about 80% complete in 8 h (Fig. 5). Furthermore, Mg^{2+}-depletion increased the rate of echinocyte formation in the presence of vanadate (Fig. 5). As expected, Ca^{2+} treatment of permeabilized erythrocytes rapidly transformed them to echinocytes, the transformation progressively increasing from 10–100 μM and reaching a maximum within a few minutes at the highest Ca^{2+} concentration (100 μM) (Fig. 6). Mg^{2+}-depletion enhanced the ability of Ca^{2+} to induce echinocytosis, except for the already very rapid rate occurring at the highest Ca^{2+} concentration examined (100 μM) (Fig. 6).

DISCUSSION

Mg^{2+}-Dependent Phosphate Production in Intact Cells

Despite the widespread distribution of Mg^{2+}-ATPase activity in plasma membranes of eukaryotic cells [25,28], few studies have characterized this activity in intact cells and attempted to ascribe a function to it. In the present study we have determined some of the basic properties of Mg^{2+}-dependent phosphate production and ATP breakdown in the intact human red blood cell and examined the possibility that this activity, likely to represent plasma membrane Mg^{2+}-ATPase, is associated with the maintenance of the normal biconcave (discocyte) shape of the intact red blood cell. Mg^{2+}-dependent phosphate production in the intact cell was about 5–10% of the Ca^{2+} plus Mg^{2+}-stimulated activity [see 18], which is similar to the ratio of Mg^{2+}-ATPase to (Ca^{2+} + Mg^{2+})-ATPase activity in membranes isolated from these cells (results not shown). Mg^{2+}-dependent phosphate production in previously Mg^{2+}-depleted cells (Fig. 1) was half-maximally activated at around 0.4 mM free Mg^{2+}, which is about the free intracellular Mg^{2+} concentration found in intact erythrocytes [29] and therefore could allow sensitive control of Mg^{2+}-ATPase activity in response to small changes in intracellular Mg^{2+} levels. Although Mg^{2+}-ATPase activity has been reported in the cytosolic [30] and skeleton fractions of red cells [31], the activities of these are 100- to 1000-fold less than that of the membrane-associated Mg^{2+}-ATPase. Therefore Mg^{2+}-dependent phosphate production in intact cells mainly reflects the Mg^{2+}-ATPase activity of the plasma membrane rather than the cytosolic fraction. While the breakdown of other organic phosphate intermediates contributes to the estimate of total phosphate production, even the hydrolysis of 2,3-bisphosphoglycerate, a major source of such phosphate generation, can account for only a small fraction of the phosphate production in normal intact cells [23].

Effects of Vanadate on ATP Hydrolysis and Red Cell Shape

It proved more difficult at first to establish the vanadate sensitivity of the Mg^{2+}-dependent ATP hydrolytic activity in the intact red cell using phosphate production as the assay, despite the fact that vanadate clearly enters the red blood cell [21,22]. Although vanadate affects a number of enzymes, including phosphatases and adenylate cyclase, and can affect red cell metabolism at high concentrations [32,33], it has been shown to be a potent inhibitor of various ATPases [15]. Under conditions similar to those used

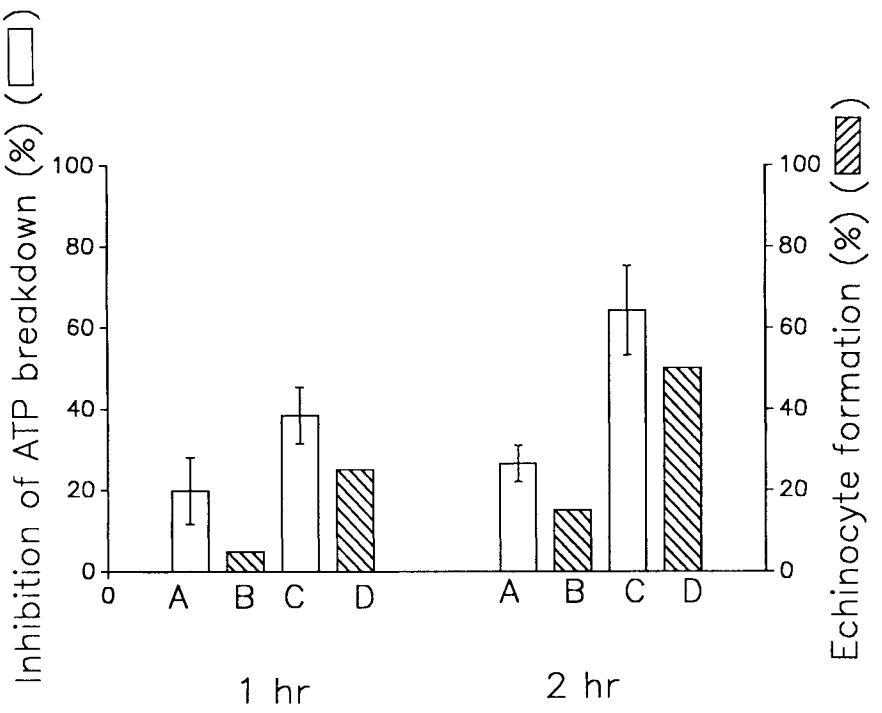

Fig. 4. Effect of vanadate on ATP hydrolysis and echinocyte formation in intact red blood cells. Washed red blood cells were incubated with either 30 μM (A, B) or 100 μM (C, D) ammonium vanadate for 1 h (**left**) or 2 h (**right**). ATP breakdown (open bars) and echinocyte formation (hatched bars) were determined as described in the Methods. The ATP levels in the controls used to calculate inhibition of ATP breakdown were 1.45 ± 0.05 mM at zero time, 0.43 ± 0.04 mM at 1 h, and 0.23 ± 0.02 at 2 h. Results represent the mean ± standard deviation from three separate experiments. Echinocyte formation was obtained from the results in Figure 2.

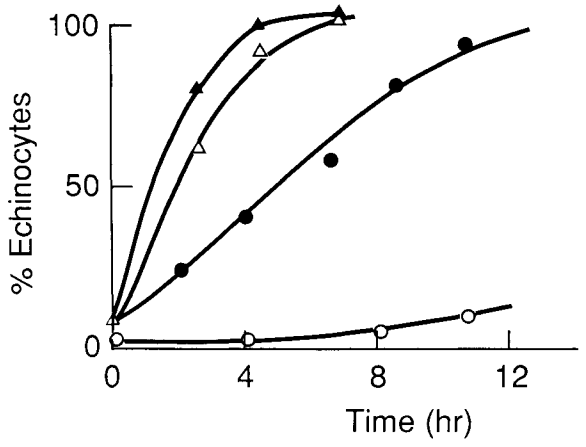

Fig. 5. Influence of vanadate on echinocyte formation in Mg^{2+}-depleted and normal intact red blood cells. Washed red blood cells (open symbols) or Mg^{2+}-depleted red blood cells (closed symbols) were incubated in the absence of vanadate (circles) or in the presence of 100 μM vanadate (triangles) at 37°C for the times shown. The percentage of echinocyte formation was determined as in Methods.

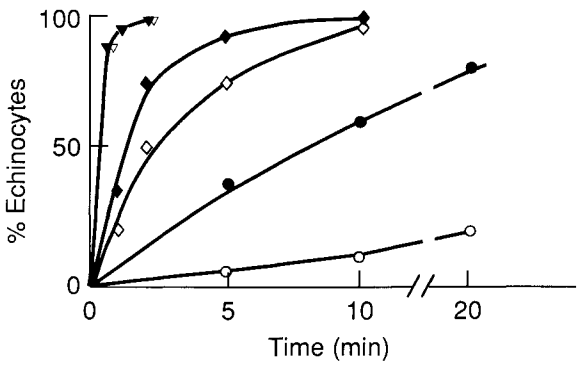

Fig. 6. Effect of Ca^{2+} loading on echinocyte formation in normal and Mg^{2+}-depleted red blood cells. Normal cells (open symbols) or Mg^{2+}-depleted cells (closed symbols) were washed and incubated with A23187 for various times with 10 μM $CaCl_2$ (circles), 30 μM $CaCl_2$ (diamonds), and 100 μM $CaCl_2$ (triangles) and echinocyte formation determined as described in Methods.

here, we have previously shown that vanadate completely inhibits the Ca^{2+}-dependent ATP hydrolytic activity associated with the active Ca^{2+}-pump in intact red blood cells [18]. Working here with the Mg^{2+}-ATPase we were able to demonstrate its vanadate sensitivity after 2,3-bisphosphoglycerate breakdown was inhibited with tetrathionate (2 mM) and inorganic phosphate levels were decreased following incubation of cells with inosine (10 mM) (Fig. 3). Given the structural homology between vanadate and phosphate undergoing hydrolysis, both adopting trigonal bipyramidal structures [34], this is not surprising, since phosphate competes with vanadate for transport into cells and for binding sites on enzymes [see 22]. Reduction of VO_3^- to VO^{2+} (vanadyl) in the presence of glutathione [35] and binding to cytoplasmic components [22] may also have contributed to the resistance of Mg^{2+}-dependent phosphate generation to vanadate in the intact cell, as in contrast to [38] we have found that VO^{2+} has no effect itself on Mg^{2+}-ATPase activity (Xu and Roufogalis, unpublished results). Such mechanisms have been proposed to explain a similar discrepancy reported with respect to the lack of effect [36] or low sensitivity [22] of vanadate for the Na^+, K^+-ATPase activity in intact cells. However, why these mechanisms do not appear to interfere with the inhibition by vanadate of the Ca^{2+}-pump in intact red cells [18] is unclear.

Inhibition of Mg^{2+}-dependent ATP hydrolysis by vanadate could be demonstrated in red blood cells without requiring their preincubation with inosine. The degree of inhibition by vanadate in these experiments, in which ATP resynthesis was blocked by pretreatment of the cells with iodoacetic acid [39], was similar to that in experiments where phosphate production was determined. After 1 h, ATP hydrolysis was inhibited by 20% at 30 μM vanadate; this increased to 38% at 100 μM vanadate and correlated with an increase of echinocyte formation from 3% to 25% (Fig. 4). Similar correlation was obtained after 2 h, when the inhibition of ATP breakdown was increased from 30% to 70% by an increase of vanadate from 30 μM to 100 μM and echinocyte formation increased from 20% to 50% (Fig. 4). Thus, there appears to be a good correlation between vanadate inhibition of Mg^{2+}-dependent ATP hydrolysis and echinocyte formation in intact red blood cells.

Effects of Magnesium and Calcium on Red Cell Shape

A number of other findings were also consistent with the association of Mg^{2+}-dependent ATP hydrolysis and shape regulation in intact cells. Thus, Mg^{2+} depletion, which inhibited Mg^{2+}-dependent phosphate production, accelerated the rate of echinocyte formation and, in turn, vanadate-induced echinocyte formation was more rapid in Mg^{2+}-depleted red cells than in normal cells (Fig. 5). Mg^{2+} depletion also enhanced the susceptibility of red blood cells to Ca^{2+}-induced echinocyte formation at Ca^{2+} concentrations which were suboptimal for inducing echinocytes (Fig. 6). Although the mechanism of Ca^{2+}-induced echinocytosis is still largely unclear, it is possible that Ca^{2+} inhibits the vanadate-sensitive Mg^{2+}-ATPase activity [38], as we previously demonstrated for Cd^{2+} in membranes (Xu and Roufogalis, unpublished results) and for Ca^{2+} on solubilized Mg^{2+}-ATPase activity (Morris and Roufogalis, unpublished results). The mechanism by which membrane Mg^{2+}-ATPase activity may control red blood cell shape remains unknown. An ATP-driven conformational change of the Mg^{2+}-ATPase during its reaction cycle may serve to couple membrane bilayer and skeleton interactions [37] by modulating the flexibility or mobility of other proteins in the bilayer and/or the skeleton. Alternatively, the Mg^{2+}-ATPase activity may be the enzyme regulating aminophospholipid asymmetry [38], thereby modulating red blood cell shape.

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