Studies on the Effect of Vanadate on Endocytosis and Shape Changes in Human Red Blood Cells and Ghosts

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When amphipathic cationic drugs are added to intact human RBCs, the RBCs first undergo a stomatocytic shape change and then, if relatively large amounts of drug are added and if the metabolic state of the RBC is appropriate, endocytic vacuoles form. Vanadate has a structural similarity to the transition state of phosphate, which presumably accounts for its ability to inhibit phosphohydrolases, although other actions of vanadate have been described. Vanadate inhibited three forms of drug-induced endocytosis in intact RBCs despite the fact that the three drugs chosen (primaquine, chlorpromazine, and vinblastine) are known to have differing requirements for RBC ATP. Vanadate also inhibited the stomatocytic shape change produced by primaquine, chlorpromazine, and vinblastine, but not the stomatocytosis produced by low pH. Vanadate had no effect on RBC echinocytosis produced by lysophosphatidylcholine. In studying endocytosis in hypotonic, leaky, “white” ghosts, we discovered that vanadate inhibited only the endocytosis produced by Mg-ATP and not the endocytosis produced by manipulations that directly attack the cytoskeletal proteins. These findings suggest that ATP hydrolysis has a role in some forms of amphipathic cation-induced stomatocytosis and endocytosis in intact RBCs. In addition, studies in ghosts support the idea that Mg-ATP does indeed produce “energized” endocytosis dependent on utilization or hydrolysis of ATP.

When amphipathic anions and cations are added to RBCs in vitro, they produce, respectively, echinocytosis and stomatocytosis. Somewhat similar shape alterations also occur under certain circumstances in vivo. Stomatocytosis can be extended in vitro to proceed to the formation of endocytic vacuoles, which are inside-out portions of the RBC plasma membrane that form preferentially in the invaginated portion of the cup. The irreversible loss of membrane surface area into endocytic vacuoles reduces the surface area/volume ratio and results in the formation of a spheroctocytocyte. Continuation of the process results in RBC lysis. A role of RBC metabolism in this in vitro process was deduced from the observation that glucose deprivation could prevent primaquine-induced hemolysis. Some forms of drug-induced endocytosis (ie, that produced by chlorpromazine) can occur in ATP-depleted RBCs, an observation that tended to uncouple the relationship between RBC metabolism and drug-induced RBC shape change. Subsequently, it was noted that most forms of drug-induced stomatocytosis and endocytosis in vitro take place more readily in glucose-supplemented, ATP-replete RBCs. It has been suggested that ATP has an important but poorly understood role in controlling intact RBC shape. On the basis of experiments performed with ghosts, a shape-mediating role of Mg-ATP acting through the membrane Mg²⁺-ATPase has been proposed.

These observations led us to suspect that there is a role for ATP and perhaps ATP hydrolysis in drug-induced stomatocytosis and the subsequent endocytosis in intact RBCs. An opportunity for evaluating the potential role of ATP hydrolysis in RBC stomatocytosis and endocytosis was provided by recent observations on the properties of the several forms of vanadium. Ionic vanadium, under certain circumstances, has a structural similarity to the transition state of phosphate, thereby perhaps accounting for its ability to inhibit phosphohydrolases at micromolar concentrations. Vanadate inhibition of RBC membrane Na⁺, K⁺-ATPase and Ca²⁺, Mg²⁺-ATPase has been reported, whereas incubation of vanadate with intact RBCs has inhibited the sodium pump in a manner consistent with inhibition of the pump-related RBC membrane Na⁺, K⁺-ATPase. Therefore, we tested a hypothesis that ATP hydrolysis was important in drug-induced stomatocytosis and endocytosis in intact human RBCs by studying the effect of vanadate on RBC shape change and endocytosis. In addition to inhibiting the phosphohydrolases, vanadate can inhibit phosphatases, stimulate adenylyl cyclase and, at very high concentrations, can produce RBC metabolic alterations even and even cause shape changes. Therefore, experiments using vanadate must be carefully controlled and interpreted.

Use of vanadate also offered us the opportunity of testing a hypothesis on endocytosis produced in white ghosts. We and others had identified two forms of endocytosis in white ghosts. One form, called “nonenergized” endocytosis, is produced by hypotonicity, EDTA, or trypsin, all of which appear to act by stripping away the cytoskeleton. The other form is produced by addition of Mg-ATP and has been called “energized endocytosis,” although the mechanism of energization has not been identified. If vanadate blocked Mg-ATP-induced energized endocytosis, it would mean that ATP hydrolysis was probably required.

MATERIALS AND METHODS

All reagents used were reagent grade or better. Sodium orthovanadate was obtained from Fisher Scientific Company, and its concentration was verified. The solutions were adjusted to pH 7.5, and only solutions that were clear and devoid of color were used. Carrier-free ³²Pi was obtained from New England Nuclear Corp (Boston). Lysophosphatidylcholine was obtained from Sigma Co, St Louis. Trypsin was obtained from the Worthington Chemical Co.
The protein concentration was determined by the method of Lowry, previously described, using three washes of 5 mmol/L of phosphate buffer described.22 Silicon 550 Fluid was obtained from Dow Corning.

Freshly drawn, heparinized venous blood was obtained from normal human donors according to a protocol approved by the Stanford University Committee on Human Experimentation. The blood was centrifuged at room temperature, and the plasma and buffy coat were removed, after which the RBCs were washed once with 15- to 20-fold volumes of 0.15 mol/L of NaCl and then three times with either cold Hanks’ solution or phosphate-buffered saline (PBS) pH 7.4 containing 2 mg glucose/mL.

**Ghost preparation.** White ghosts were made exactly as previously described, using three washes of 5 mmol/L of phosphate buffer pH 8.0 at a ratio of 40 vol of lysis solution to 1 vol of packed RBCs.22 The protein concentration was determined by the method of Lowry, using five times recrystallized bovine serum albumin (BSA) as standard.

**ATPase assays.** The several different ATPase activities displayed by ghosts were measured exactly as previously described using γ-32P-ATP as substrate.22 The 32Pi generated as a result of ATPase action was quantitatively recovered in the isobutanol-benzene extract.22 Four ATPases were measured. All assays contained ∼100 µg of membrane protein, 2.5 mmol/L of MgCl2, and 1.5 mmol/L of NaCl-γ-32P-ATP, which defined the Mg2+-ATPase. For the Na+-, K+-ATPase, 30 mmol/L of KCl and 100 mmol/L of NaCl were added. For the Ca2+-Mg2+-ATPase, 0.12 mmol/L of CaCl2 was added. For the actin-activated ATPase, 100 µg of RBC membrane actin was added to 100 µg of RBC membrane protein.

**RBC endocytosis.** Endocytosis was induced in intact RBCs by adding primaquine, chlorpromazine, and vinblastine as described above.24 Four ATPases were measured. All assays contained ∼100 µg of membrane protein, 2.5 mmol/L of MgCl2, and 1.5 mmol/L of NaCl-γ-32P-ATP, which defined the Mg2+-ATPase. For the Na+-, K+-ATPase, 30 mmol/L of KCl and 100 mmol/L of NaCl were added. For the Ca2+-Mg2+-ATPase, 0.12 mmol/L of CaCl2 was added. For the actin-activated ATPase, 100 µg of RBC membrane actin was added to 100 µg of RBC membrane protein.

**Endocytosis in white ghosts.** Endocytosis was induced in intact RBCs by adding primaquine, chlorpromazine, and vinblastine as described previously.24 To test the effect of vanadate on RBC endocytosis, it was preincubated with RBCs suspended in plasma or Hanks’ solution for 30 to 60 minutes at 37 °C to allow passage of vanadate into the RBC interior.22 At the end of the preincubation period, the endocytosis-inducing drug was added, and the timing of the incubation was begun and continued at 37 °C for the length of time indicated in the text and Tables 1 through 6. The extent of endocytosis was monitored by phase microscopy and by the radiotopic method, which is based on the trapping of 57Co-vitamin B12 within endocytic vacuoles.24 Preliminary experiments indicated that vanadate, at the concentrations used (5 to 100 µmol/L), produced neither RBC shape changes nor endocytosis, nor did it interfere with the binding of the 57Co-vitamin B12-transcobalamin complex to the RBC surface. ATP levels were measured by the coupled hexokinase-G6PD assay22 in intact RBCs before and after incubation with the endocytosis-inducing drugs alone or in combination with vanadate. It was important to determine whether the inhibitory effects of vanadate were reversible. Therefore, RBCs as described above were incubated without and with vanadate at 37 °C. Then, relying on the published information of vanadate efflux,21 we washed the RBCs twice with a 20-fold volume of Tris-buffered saline pH 7.2 containing 1% BSA. We then incubated them overnight at 37 °C at a hematocrit of 10% in Hanks’ solution containing 10% autologous plasma, 5 mmol/L of glucose, and penicillin 200 U/mL and streptomycin 200 µg/mL. In the morning, the RBCs were washed again three times with Hanks’ solution and were then studied as above for their capacity to undergo cationic amphipath-induced RBC shape change and endocytosis.

**RBC shape changes.** To produce echinocytosis, intact washed RBCs were incubated in PBS-glucose (2 mg/mL) at 2% hematocrit with 6 µg/mL of lysophosphatidylcholine for up to 30 minutes at 37 °C without or with the addition of vanadate. Samples of 20 µL were removed at given time intervals from 5 to 30 minutes, added to 200 µL of 1% glutaraldehyde in PBS, and then examined by phase microscopy. The proportion of RBCs of different morphologies was recorded using the nomenclature and classification of Bessis.2 The effect of vanadate on shape change reversibility was then measured by removing the suspending media and washing the RBC pellet serially with either 1% BSA in PBS, or 1% BSA plus 30 µmol/L of vanadate in PBS. Morphologic assessment by phase microscopy was made of the RBCs after each wash. Selected samples were also photographed by Nomarski interference microscopy. The effect of vanadate on induction of stomatocytosis was also studied. The amphipathic cations, primaquine, chlorpromazine, and vinblastine, were used to produce stomatocytosis; the incubations were carried out as described for endocytosis. Acid-pH was also reported by Deuticke21 to induce stomatocytosis. Therefore, washed intact RBCs at a hematocrit of 2% were incubated in PBS pH 7.4 as a control, and then with both 0.1 mol/L of PO4 buffer pH 5.2 or citrate buffer 0.1 mol/L pH 5.2 at 37 °C for 30 minutes without or with concurrent addition of 30 µmol/L of vanadate. Samples were obtained for morphologic analysis. Reversal of shape change was then performed by washing either with 1% BSA in PBS pH 7.4 or 1% BSA plus 30 µmol/L of vanadate in PBS pH 7.4.

**Drug entry into RBCs.** The effect of vanadate on the uptake of chlorpromazine and vinblastine was measured by a method previously used, with a single modification.23 Instead of diluting and washing the RBC pellet after incubation, we layered the samples on top of a 1-mL silicon barrier in a 1.5-mL Eppendorf centrifuge tube and immediately centrifuged them at room temperature for 2 minutes at full speed. The clear supernatant formed a distinctly visible band above the barrier, and the RBC pellet was densely packed at the bottom of the tube. The supernatant fraction was carefully removed and added to a scintillation vial. The RBC pellet was harvested by cutting off the tip of the tube and placing it in a scintillation vial. One milliliter of Protosol/ethanol (1:2) was added to all vials, which were digested at 60 °C for 90 minutes and then allowed to cool, after which 0.3 mL of 30% H2O2 was added and the samples were stored overnight at room temperature. Ten milliliters of Biofluor was added, and the vials were shaken; then 0.5 mL of 0.5 N HCl was added. The mixture was shaken, allowed to cool for 2 hours, and then counted by liquid scintillation spectrometry. The specific activity of endocytosis-inducing drugs was adjusted by adding radioisotopes to “cold” drugs to provide a stock standard solution. The chlorpromazine specific activity was used as 1.13 x 107 cpm/µmol, and the vinblastine specific activity was 3.46 x 107 cpm/µmol.

**Ghost endocytosis.** Endocytosis in white ghosts was induced by three methods.24 Addition of 20 µg/mL of trypsin or 0.1 mmol/L of EDTA produces endocytic vacuoles presumably by stripping off segments of the cytoskeletal proteins, i.e., nonenergized endocytosis. Conversely, addition of 3 mmol/L of Mg-ATP to leaky ghosts suspended in 50 mmol/L of TES pH 7.5 produces energized endocytosis.24 The extent of endocytosis was measured semiquantitatively by observing coded ghosts samples under phase microscopy. The specific activity of endocytosis-inducing drugs was adjusted by adding radioisotopes to “cold” drugs to provide a stock standard solution. The chlorpromazine specific activity was used as 1.13 x 107 cpm/µmol, and the vinblastine specific activity was 3.46 x 107 cpm/µmol.

**Radiophosphorylation studies of ghosts during endocytosis.** In attempts to explore the molecular mechanism by which Mg-ATP...
might cause ghost endocytosis, RBCs were radiolabeled by incubating them under sterile conditions for 20 hours with 100 to 500 µCi of 32P/mL packed RBCs. Ghosts were then prepared and incubated as above with 50 mmol/L of TES buffer, and 3.0 mmol/L of Mg-ATP without and with the addition of 30 µmol/L of vanadate to block endocytosis. Following incubation at 37 °C for a time sufficient to cause extensive endocytosis in the samples free of vanadate, ghosts were separated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on slab gels using the Laemmli system as previously described. The gels were analyzed by densitometry, bands were cut out and weighed, and the proportion of total proteins was recorded. The extent of radiophosphorylation of the membrane proteins was then assessed semiquantitatively by 32P-radioautography and quantitatively by slicing out the relevant bands, dissolving them in 600 µL of 30% H2O2, adding aquasol, and measuring the radioactivity by liquid scintillation spectrometry. From the radioisotopic data and protein concentrations, the 32P specific activity of the individual phosphorylated proteins could be calculated.

RESULTS

ATPase assays. The ATPase assays were carried out in duplicate as described in the Materials and Methods section, and the samples were incubated 60 min at 37 °C both without vanadate and with the addition of 2 and 20 µmol/L of vanadate at the start of the incubation period. The results (Table 1) showed that all the ATPases were strongly inhibited by vanadate at 20 µmol/L and that Mg2+-ATPase showed the greatest sensitivity, being inhibited 53% at 2 µmol/L of vanadate. Ghost endocytosis and the effect of vanadate. Experiments were designed to test the effect of vanadate on energized endocytosis as produced by addition of 3 mmol/L of Mg-ATP, or on nonenergized endocytosis produced by incubation of ghosts with 20 ng/mL of trypsin or 0.1 mmol/L of EDTA. Incubation was carried out (as described in the Materials and Methods section) at 37 °C, with samples observed at 0, 15, 30, 45, and 60 minutes. In each case, a sample with buffer alone and another with an appropriate concentration of vanadate used were incubated in parallel. Acetylcholinesterase results were correlated with independent coded morphologic observations. Vanadate in concentrations of 30 µmol/L had no inhibitory effect on endocytosis produced by either EDTA or trypsin. In contrast, it profoundly inhibited Mg-ATP endocytosis (Table 2A). This effect of vanadate was completely reversible. Ghosts incubated with 30 µmol/L of vanadate were washed three times with 50 mmol/L of TES, after which their ability to undergo Mg-ATP-induced endocytosis was unimpaired as compared with values of ghost suspensions incubated in parallel but without prior vanadate addition (Table 2B). Ghosts were then prepared from RBCs that had been radiophosphorylated. Aliquots were taken for analyses at 0, 60, and 150 minutes of incubation at 37 °C. No differences in the amount and distribution of the membrane proteins were detectable by densitometry; nor were there detectable differences in the phosphorylation patterns of the membrane proteins among four samples containing: buffer only; 30 µmol/L of vanadate; 3 mmol/L of Mg-ATP, and 3 mmol/L of Mg-ATP plus 30 µmol/L of vanadate (data not shown).

Effect of vanadate on drug-induced endocytosis. Addition of up to 100 µmol/L of vanadate and incubation for up to 60 minutes at 37 °C had no effect on RBC shape or on the binding of the indicator 57Co-vitamin B12-transcobalamin complex to the RBC outer surface (data not shown). Vanadate was allowed to enter RBCs during a 60-minute preincubation at 37 °C. Endocytosis produced by all three drugs (Table 3) was inhibited by vanadate. The magnitude of the vanadate effect can be appreciated from a single experiment, in which primaquine endocytosis trapped 1,150 cpm of 57Co/10¹⁰ RBCs but preincubation with 30 µmol/L of vanadate reduced that to 250 cpm/10¹⁰ RBCs. In the same experiment, control values for chlorpromazine endocytosis were 881 cpm/10¹⁰ RBCs, falling to 313 cpm/10¹⁰ RBCs on addition of 30 µmol/L of vanadate. Parallel values for

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<th>Table 1. Effect of Vanadate on RBC Membrane ATPases</th>
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<td>Vanadate Concentration (µmol/L)</td>
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Number of experiments in parentheses. Values are expressed as percent of inhibition of the control value; mean ± SD.
vanblaste were 1,781, falling to 619. Phase microscopy revealed that >50% of RBCs had one or more vacuoles in control cationic amphipath-treated samples, whereas after vanadate <10% of the RBCs contained vacuoles except in the vanblaste sample, in which ~20% of the RBCs contained vacuoles. Primaquine endocytosis seemed most susceptible, with an $I_{sp}$ of ~7.5 $\mu$mol/L of vanadate.

Because it is known that reduction in ATP levels can impair drug-induced endocytosis, particularly primaquine endocytosis, six experiments were performed with six different donors to measure ATP levels in RBCs exposed to endocytosis-inducing drugs without or with the addition of vanadate. Table 4 shows the results of a single experiment and indicates that no change in ATP levels occurred as a consequence of incubation with amphipathic cations or vanadate.

**Effect of vanadate on RBC shape change.** Because our experimental design called for coded morphologic assessment of RBC shapes during drug-induced endocytosis, it became apparent that vanadate not only blocked endocytosis but also blocked the acquisition of the spherostomatocytic shape change that invariably precedes endocytosis (Fig. 1). Figure 1 shows the results with primaquine; however, vinblaste and chlorpromazine stomatocytosis were similarly blocked. Primaquine and vinblaste produce the stomatocytic shape change over a period of minutes, whereas the chlorpromazine shape change is virtually instantaneous. Nevertheless, preincubation with vanadate blocked the chlorpromazine-induced stomatocytosis (data not shown) as effectively as it blocked primaquine and vinblaste stomatocytosis. Higher concentrations of primaquine (4 to 6 mmol/L) could partially overcome the shape inhibition of 30 $\mu$mol/L of vanadate (data not shown) but not of 100 $\mu$mol/L of vanadate.

The next question was whether vanadate could block other forms of shape change. Acid pH produces stomatocytes I and II. We therefore incubated RBCs as described in the Materials and Methods section without or with vanadate in isotonic citrate or phosphate buffer at pH 5.2. At intervals, RBCs were removed, fixed with glutaraldehyde, and examined under phase microscopy. In two such experiments, vanadate had no effect on the acquisition of the stomatocytic shape produced by acidic buffers (data not shown). The shape change produced by acid pH was not readily reversed by four 60-fold volume washes in 1% BSA dissolved in PBS pH 7.4. Therefore, the role of vanadate on the reversal of the acid pH stomatocytic shape change could not be assessed.

Echinocytosis was induced as described in the Materials and Methods section by adding 6 $\mu$g/mL of lysophosphatidylcholine, which produces echinocytes II and III. Addition of 30 $\mu$mol/L of vanadate had no effect on the appearance or extent of this shape change. The reversibility of this shape change was then studied by washing RBCs in 1% BSA in PBS; neither preincubation with vanadate nor addition of vanadate to the wash solution interfered with the prompt reversal of the echinocytic shape change to the discocytic shape.

**Reversibility of vanadate effect on RBC stomatocytosis and endocytosis.** To determine if the effect of vanadate was reversible, RBCs that had been pretreated with 30 $\mu$mol/L of vanadate were extensively washed and then incubated overnight under conditions calculated to enhance vanadate efflux. The results of one of two such experiments indicated (Table 5) that the effect of vanadate on endocytosis was reversible. The effects of vanadate on inhibition of stomatocytosis were also completely reversible (data not shown).

**Effect of vanadate on drug entry into RBCs.** It was possible that micromolar amounts of vanadate could block uptake of amphipathic drug into RBCs. Therefore, the effect of vanadate on drug uptake was measured using identical

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<th>Table 4. RBC ATP Levels</th>
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<td><strong>Value</strong></td>
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<td>Initial RBC ATP value</td>
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<td>Following incubation with Vanadate 30 $\mu$mol/L and:</td>
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<td>No drug added</td>
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<tr>
<td>Primaquine 3 mmol/L</td>
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<td>Chlorpromazine 1 mmol/L</td>
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<td>Vinblaste 0.6 mmol/L</td>
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Values are $\mu$mol ATP/mL packed RBCs.

| Table 5. Reversibility of Vanadate Inhibition of RBC Endocytosis |
|----------------------|------------------------|
| **Drug** | Fresh RBCs Treated With Vanadate 30 $\mu$mol/L (%) | Vanadate-Treated (30 $\mu$mol/L) RBCs, Washed and Incubated Overnight (%) |
| Primaquine | 3 mmol/L | 76 | 5 |
| Chlorpromazine | 1 mmol/L | 31 | 0 |

Values are percentage of inhibition.
concentrations and ratios of drug, RBCs, buffer, and plasma, and the same time intervals we used to study endocytosis. Tritiated radioisotopes of vinblastine and chlorpromazine are available and we have previously studied their uptake into RBCs. The recoveries of radioisotopic drug were in the order of 80%. Vanadate produced no difference in RBC uptake of either chlorpromazine or vinblastine. One of the two experiments performed is shown in Table 6.

**DISCUSSION**

The chemistry of vanadium and the oxyvanadium compounds has been the object of considerable study because of their potential physiological role. At nanomolar to micromolar concentrations and at physiologic pH, the predominant species is pentavalent metavanadate anion (VO$_3^-$). The metavanadate may resemble the transition state of phosphate, thus allowing it to insert into phosphate subsites of some ATP binding sites. Perhaps it is by this mechanism that metavanadate inhibits the RBC membrane Na$^+$, K$^+$-ATPase, and Ca$^{2+}$-ATPase, as well as various phosphohydrolases in kidney, muscle, nerve, and liver. These studies generally have been carried out on disrupted cell fractions. Vanadate can also inhibit the Na$^+$-K$^+$ pump when added to intact RBCs, but somewhat higher concentrations are required. One explanation is that vanadate transverses the RBC membrane as the metavanadate anion; once inside the RBCs, however, substantial amounts of metavanadate are reduced to the tetravalent vanadyl cation (VO$_2^+$) that is less inhibitory to the Na$^+$, K$^+$-ATPase and may that also bind to cellular constituents.

In addition to its activity against ATPases, vanadate can also inhibit phosphatases, stimulate adenylyl cyclase, and inhibit ATP-dependent proteases. Of importance to our study is the fact that vanadate at the micromolar concentrations used (2 to 100 μmol/L) has no effect on RBC shape, deformability, osmotic fragility, or metabolism, whereas it can affect all these modalities at considerably higher concentrations.

We initially confirmed reports that micromolar concentrations of vanadate inhibited four human RBC membrane ATPases: Mg$^{2+}$-ATPase, Na$^+$-$K^+$-ATPase, Ca$^{2+}$-ATPase, and actin-activated ATPase (Table 1). The membrane preparation used for these measurements is essentially the same as the leaky hypotonic ghosts used to study endocytosis.

In ghosts suspended in 50 mmol/L of TES, endocytosis can be produced by 3 mmol/L of Mg-ATP (energized endocytosis) and by attack on the cytoskeleton produced by trypsin or EDTA (nonenergized endocytosis). Vanadate blocked only Mg-ATP endocytosis (Table 2), thereby suggesting that hydrolysis of ATP is the important factor in energized endocytosis. Fairbanks and colleagues noted that vanadate blocked the discocytic shape transformation produced by isotonically sealing Mg-ATP within ghosts and suggested that vanadate inhibited Mg$^{2+}$-ATPase, which could be a shape-change–mediating enzyme. The evidence linking Mg$^{2+}$-ATPase to ghost shape change is based on a parallelism between the concentration of vanadate that inhibits Mg$^{2+}$-ATPase and the concentration of vanadate that inhibits Mg-ATP–induced ghost discocytosis.

Using a similar form of analysis, we suggested that in a somewhat different preparation—sealed red ghosts—endocytosis requires the Ca$^{2+}$-induced activation of Ca$^{2+}$, Mg$^{2+}$-ATPase, and that in the absence of Ca$^{2+}$ or in the presence of inhibitors of Ca$^{2+}$, Mg$^{2+}$-ATPase red ghost endocytosis was inhibited. Hayashi and Penniston used a series of alkylating agents and noted a strong correlation between inhibition of ATPase and inhibition of endocytosis in ghosts. They proposed that inhibition at a single site accounted for both actions.

In trying to determine which of the ATPases might be involved, they noted concurrent inhibition of ghost endocytosis and a low-affinity Ca$^{2+}$-stimulated Mg$^{2+}$-ATPase. It is unlikely, however, that there is any free Ca$^{2+}$ in these thoroughly washed ghosts. Thus, the Mg$^{2+}$-ATPase as well as the actin-activated ATPase are candidates for the mechanisms by which Mg-ATP could energize, and vanadate block, ghost endocytosis. The prompt reversibility of the vanadate effect is consistent with the reversibility of vanadate inhibition of RBC membrane ATPases and against the idea that vanadate caused the oxidation of sulfhydryl groups. Attempts to identify a membrane protein dephosphorylated during Mg-ATP–induced endocytosis were unsuccessful. The methods used, however, were suitable only to detect dephosphorylation that might have occurred in β spectrin, protein 2.1, 3, or 4, but not the RBC membrane ATPases. In addition to ATPase action, the hydrolysis of Mg-ATP in ghost endocytosis may be produced by protein kinases, by enzymes involved in lipid phosphorylation of ghost membranes, or by as yet unknown mechanisms.

To study endocytosis in RBCs, a very different system than leaky ghosts, we preincubated intact RBCs with vanadate at concentrations reported to inhibit the Na$^+$-K$^+$ pump and presumably the pump-related Na$^+$-$K^+$-ATPase; we observed a dose-dependent inhibition of drug-induced endocytosis (Table 3). It is highly unlikely that the RBC membrane Na$^+$, K$^+$-ATPase was involved in drug-induced...
endocytosis since neither ouabain addition nor substitution of choline for Na+ or K+ have an effect on such endocytosis. We have previously shown that primaquine-induced endocytosis is absolutely dependent on active glycolysis and maintenance of ATP levels, whereas vinblastine and chlorpromazine endocytosis are reduced ~60% but not obliterated by RBC ATP depletion.6 Preincubation of RBCs with vanadate did not reduce RBC ATP levels (Table 4). Because ATP was not depleted and micromolar additions of vanadate inhibited endocytosis, it is highly likely that it is not ATP in itself, but ATP hydrolysis that is involved in primaquine endocytosis to a great degree and in chlorpromazine and vinblastine endocytosis to a lesser extent.

While we were monitoring endocytosis morphologically, it quickly became apparent that the stomatocytosis (Fig 1) that invariably precedes the endocytosis was blocked by vanadate. We confirmed the fact that vanadate at the concentrations used (2 to 100 μmol/L) did not cause echinocytosis. Therefore, its action in blocking the stomatocytic shape change was not to produce a conflicting or neutralizing shape alteration. We then addressed the question of whether vanadate blocked all forms of stomatocytosis or only those produced by cationic amphipaths. The stomatocytosis produced by pH 5 buffers was not inhibited by vanadate. The next question was whether vanadate would inhibit all forms of amphipath-induced shape change. Vanadate did not inhibit the echinocytosis produced by incubation of intact RBCs with lysophosphatidylcholine.

The use of vanadate indicates that all forms of stomatocytosis do not proceed by identical mechanisms because acid pH stomatocytosis is not blocked by vanadate but cationic amphipath-induced stomatocytosis is. The initial description of the bilayer couple hypothesis suggested that amphipath-produced stomatocytosis and echinocytosis were, in a sense, mirror images, with the charge of the amphipath passively determining its localization in the lipid bilayer and thus the site of membrane expansion. Our studies show, however, that micromolar amounts of vanadate (metavanadate and vanadyl) block the RBC stomatocytic shape change produced by cationic amphipaths but not lysophosphatidylcholine-induced echinocytosis. A possible explanation for this difference comes from recent data indicating that spin-labeled phospholipids may assume their transbiliary asymmetric distribution in resealed RBC ghosts by a mechanism using Mg-ATP and blocked by 1 to 2 μmol/L of vanadate.7 In intact RBCs, Mg-ATP is required to translocate amino-phospholipids to the inner half of the bilayer.8 Thus, vanadate could block Mg-ATP-mediated translocation of cationic amphipaths to the inner half of the bilayer. Against this proposal are the data in Table 6 indicating that vanadate does not inhibit the passage of such drugs through the membrane, during which time they would have access to the inner half of the bilayer. Mg-ATP-induced translocation of drug would only be involved if such drugs passed through the membrane in sealed channels and if their subsequent access from cytosol to the inner half of the bilayer was blocked by the cytoskeleton.

These studies indicate that it is not the presence of ATP but ATP hydrolysis, reversibly blocked by vanadate, that is required for certain forms of stomatocytosis and endocytosis in RBCs and endocytosis in ghosts. It is not proven that RBC and ghost endocytosis proceed by identical mechanisms, but the vanadate inhibition of both, at similar concentrations, suggests that the two are related. The pathways by which the hydrolysis of ATP leads to these morphologic changes must be determined.

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