Erythrocyte survival depends heavily on ATP to provide energy for Na\(^{+}\),K\(^{-}\)-ATPase and Ca\(^{2+}\)-ATPase pumps and for protein phosphorylation.\(^1\) Glycolysis is the main source of ATP resynthesis from ADP in erythrocytes and phosphofructokinase (PFK) catalyzes the key regulatory step in this pathway. PFK is a tetrameric enzyme characterized by three different isoforms, muscle (M), liver (L), and platelet (P), which are encoded by a multigene family.\(^2\) Erythrocytes express both the M- and L-type PFK genes and contain five different tetramers comprised of various molar ratios of M and L subunits. Skeletal muscle contains only isofrom M homotetramers and an inherited deficiency in the M-type gene (familial phosphofructokinase deficiency) presents with exercise intolerance, muscle cramps and myoglobinuria.\(^3\) Individuals with this deficiency also exhibit a compensated hemolytic anemia\(^4\) and circulating erythrocytes have 17-30% lower steady-state levels of ATP.\(^4\) The 50% lower PFK activity in patients’ erythrocytes\(^4\) could diminish ATP production. However, glycolytic flow is not measurably altered as evidenced by a similar production of lactate in erythrocytes from patients and controls during an experimental period of energy imbalance.\(^4\) In addition, normal steady-state levels of ATP are observed in a complete deficiency of another glycolytic enzyme, glucose phosphate isomerase.\(^5\)

An alternative hypothesis for erythrocyte energy imbalance in familial phosphofructokinase deficiency is related to disturbed calcium homeostasis.\(^5\) Compared to controls, patient erythrocytes exhibit increased steady-state levels of calcium\(^2+\) and enhanced calcium loading and a loss of volume after 24 hours of autoincubation (incubation of whole blood at 37°C) in the presence of calcium.\(^6\) These observations are consistent with an increased metabolic pool of intracellular calcium and a loss of K\(^+\), Cl\(^-\) and H\(_2\)O (Gardos-effect) in patients’ erythrocytes. Therefore, it has been proposed that membrane leakage of Ca\(^{2+}\) into erythrocytes in familial phosphofructokinase deficiency results in a compensatory increase of Ca\(^{2+}\)-ATPase activity that depletes ATP and 2,3-bisphosphoglycerate and leads to diminished erythrocyte deformability and a higher rate of hemolysis.\(^3\)

Circulating erythrocytes in familial phosphofructokinase deficiency also have elevated levels of IMP.\(^7\) This observation is notable because mature erythrocytes are unable to synthesize AMP from IMP due to a developmental loss of adenylsucinate synthetase.\(^8\) These combined observations suggest that activated AMP deaminase contributes to the accelerated irreversible depletion of erythrocyte adenine nucleotides in familial phosphofructokinase deficiency. Recently, Ca\(^{2+}\)-calmodulin was shown to bind and activate erythrocyte AMP deaminase (isoform E).\(^9\) This study was designed to determine whether Ca\(^{2+}\)-calmodulin activation of isoform E contributes to the erythrocyte metabolic dysregulation in familial phosphofructokinase deficiency.

**Design and Methods**

**Patients and controls**

Three healthy control subjects and three individuals with familial phosphofructokinase deficiency were included in this study. From the Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI, USA (RLS); Department of Cardiology, Umeå University Hospital, Umeå, Sweden (AW); Department of Clinical Sciences, The University Hospital, Uppsala, Sweden (GR). Correspondence: Richard L. Sabina, Ph.D., Department of Biochemistry Medical College of Wisconsin 8701 Watertown Plank Road, Milwaukee, WI 53226, USA. E-mail: sabinarl@mcw.edu

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Preparation of erythrocyte extracts
To confirm that the patients’ circulating erythrocytes had disturbed adenine nucleotide metabolism, aliquots of fresh whole blood were centrifuged at 1020 × g for 10 minutes in a clinical centrifuge, then the majority of white cells, platelets and reticulocytes were collected by removing the plasma and buffy coat with a Pasteur pipette. The erythrocyte pellet was washed twice in 1 mM sodium phosphate, pH 7.4, containing 150 mM sodium chloride, then resuspended at a hematocrit of approximately 20% in wash buffer containing 5 mM glucose. Acid-soluble extracts were prepared as previously described \(^9\) and stored at -20°C until analysis.

Autoincubation
Autoincubation (incubation of erythrocytes in their own plasma) was used as an experimental condition of energy imbalance \(^1\) to determine whether Ca\(^{2+}\)-calmodulin activation of isoform E contributes to adenine nucleotide metabolic dysregulation in patients’ erythrocytes. Aliquots of fresh whole blood were precubated for 11 hours at 4°C with 10 mg/mL of compound 48/80, a diffusible calmodulin antagonist, or with an equivalent volume of water, which added 5% to the total volume in both cases.

Because plasma components may adsorb compound 48/80, it was necessary to use a higher concentration than those shown to inhibit intracellular calmodulin functions in other experimental systems. \(^8,10\) Three 1-mL volumes of each sample were then incubated at 37°C for 6 hours. Aliquots (200 µL) were removed at 0, 3 and 6 hours and placed immediately on ice. Neutralized acid soluble extracts were prepared as previously described \(^9\) and stored at -20°C until analysis.

Quantification of acid-soluble purine nucleotides, nucleosides and bases
Erythrocyte adenine nucleotides and IMP were separated by anion-exchange high performance liquid chromatography (HPLC) as previously described. \(^8\) Adenosine, inosine and hypoxanthine were separated on an ODS-5 column (Whatman Ltd.) by reverse-phase HPLC at a flow rate of 1 mL/minute under the following conditions (Buffer A, 150 mM sodium phosphate, pH 7.1 for 10 minutes, followed by a 30-minute linear gradient of Buffer A and Buffer B (150 mM sodium phosphate, pH 7.0, containing 25% (v/v) methanol).

All metabolites were quantified by comparison to external standards and normalized to hemoglobin, as measured in parallel aliquots of whole blood or washed erythrocytes using a commercially available kit (Pointe Scientific Inc.). Compound 48/80 was obtained from Sigma. All other chemicals were of the highest purity commercially available.

This study was approved by the ethical committee of the medical faculty at Umeå University and by the Internal Review Board at the Medical College of Wisconsin.

Results and Discussion
As previously observed, \(^7\) steady-state ATP levels were 26% lower in freshly prepared erythrocytes of patients with familial phosphofructokinase deficiency (4.02±0.57 [patient] vs 5.46±0.32 [control] µmol/g Hb; N=3, p<0.05) and IMP was 3.8-fold higher (0.45±0.06 [patient] vs 0.12±0.04 [control] µmol/g Hb; N=3, p<0.05). These values were not significantly different following preincubation of whole blood at 4°C (Figure 1, time 0 data in the absence of compound 48/80). Patients’ erythrocytes also accumulated significantly more IMP during autoincubation at 37°C (Figure 1, top panels and bottom left panel) and this difference was particularly pronounced during the first 3 hours, during which time control cells accumulated ADP and AMP, but very little IMP. Patients’ erythrocytes also had an increased IMP/AMP ratio (Figure 1, bottom right graph), indicative of a more robust AMP deaminase activity, during the first 3 hours of autoincubation. Control erythrocytes subsequently exhibited increases in IMP and the IMP/AMP ratio. These data show that erythrocyte AMP deaminase is constitutively active in familial phosphofructokinase deficiency, whereas normal cells initially maintain this enzyme in a relatively inactive state in spite of an increased substrate concentration.

In order to test the hypothesis that Ca\(^{2+}\)-calmodulin constitutively activates isoform E in familial phosphofructokinase deficiency secondary to disturbed calcium homeostasis, parallel aliquots of whole blood were exposed to a diffusible calmodulin antagonist prior to the period of energy imbalance induced by autoincubation. Additional data in Figure 1 show that compound 48/80 significantly slowed IMP accumulation (middle panels and bottom left panel) and lowered IMP/AMP ratios (bottom right graph) in patients’ erythrocytes to a point at which the levels of IMP were similar to those in untreated control cells after 6 hours of autoincubation. Moreover, this outcome was achieved in spite of a more rapid decline in ATP, likely due to the inhibition of glycolysis by the calmodulin antagonist. \(^13\) The calmodulin antagonist also slowed IMP accumulation and the increase in the IMP/AMP ratio in control erythrocytes between 3 and 6 hours, indicating that a protein-protein interaction between Ca\(^{2+}\)-calmodulin and isoform E can also occur in these cells.

Figure 2 illustrates the blood levels of hypoxanthine, the predominant diffusible AMP catabolite that accumulated during autoincubation (adenosine and inosine accounted for less than 5% of the total catabolite pool; data not shown). Control erythrocytes produced more hypoxanthine during the first 3 hours of autoincubation, suggesting that they initially utilize the alternate AMP catabolic route that proceeds through cytosolic 5’-nucleotidase (adenosine→inosine→hypoxanthine). \(^14\) Conversely, erythrocyte catabolic flow proceeds prima-
Although many µmol/g Hb IMP, mol/g Hb p+ mol/g Hb + − µ− mol/g Hb IMP, accumulated in erythrocytes of patients with familial phosphofructokinase deficiency (FPD). Top and middle: bar graph representations of erythrocyte adenine nucleotide and IMP pools (left to right, ATP, ADP, AMP and IMP) in cells from controls (left panels) and patients (right panels) in the absence (upper panels) and presence (lower panels) of compound 48/80. Bottom: line graph representation of erythrocyte IMP pools (left panel) and IMP/AMP ratios (right panel) in cells from controls (triangles) and patients (squares). Closed symbols, in the presence of compound 48/80; open symbols, in the absence of compound 48/80. Data are presented as the mean±S.D. (n=3 individuals in each group). ^p<0.05 when compared to the corresponding time 0 point in a paired Student's t-test. *p<0.05 when compared to the corresponding 3 hour time point in a paired Student's t-test. †p<0.05 when compared to the corresponding time point in the absence of compound 48/80 in a paired Student's t-test.

In conclusion, this study shows that the primary underlying mechanism for increased catabolic flow through the AMP deaminase reaction in circulating erythrocytes of individuals with familial phosphofructokinase deficiency is Ca2+ calmodulin activation of isoform E. This is the first demonstration of this regulatory mechanism in a clinical setting and lends further support to the hypothesis that disturbed calcium homeostasis is responsible for the erythrocyte energy imbalance in this compensated hemolytic anemia. The constitutive activation of isoform E, combined with the inability of erythrocytes to reanimate IMP to AMP, results in an accelerated, irreversible turnover of the erythrocyte adenine nucleotide pool. Conversely, normal erythrocytes initially use the alternate catabolic pathway that produces adenosine, which can be salvaged back into the adenine nucleotide pool. Although many factors contribute to increased hemolysis in anemic conditions, the results of this study suggest that therapeutic strategies directed against activated isoform E could be beneficial to the erythrocyte manifestations of familial phosphofructokinase deficiency.
References


