Intracellular Mg\(^{2+}\) regulates ADP phosphorylation and adenine nucleotide synthesis in human erythrocytes

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Page, Sarah, Michael Salem, and Maren R. Laughlin. Intracellular Mg\(^{2+}\) regulates ADP phosphorylation and adenine nucleotide synthesis in human erythrocytes. Am. J. Physiol. 274 (Endocrinol. Metab. 37): E920–E927, 1998.—\(^{13}\)C- and \(^{31}\)P-NMR were used in methylene blue-treated human erythrocytes to determine the dependence on intracellular Mg\(^{2+}\) concentration ([Mg\(^{2+}\)]i) of the pentose phosphate pathway (PPP), the glycolytic pathway, and adenine nucleotide synthesis. The PPP flux had an [Mg\(^{2+}\)]i at half-maximal velocity ([Mg\(^{2+}\)]i,0.5) of 0.02 mM, well below the physiological range (0.2–0.7 mM). Flux through the PPP was reduced at higher [Mg\(^{2+}\)]i as flux through phospho fructokinase was increased ([Mg\(^{2+}\)]i,0.5 = 0.16 mM). [Mg\(^{2+}\)]i,0.5 of phosphoglycerate kinase flux, which equals net ADP phosphorylation rate, was 0.27 mM, well within the physiological [Mg\(^{2+}\)]i range. The rate of adenine nucleotide synthesis from [2-\(^{13}\)C]glucose-derived ribose 5-phosphate and exogenous adenine also exhibited dependence on [Mg\(^{2+}\)]i but was not saturable up to 1.6 mM. Therefore, net flux through the PPP and glycolytic pathways in erythrocytes is not strongly dependent on [Mg\(^{2+}\)]i at physiological ion concentrations, but both ADP phosphorylation and adenine nucleotide synthesis are likely to be regulated by normal fluctuations in [Mg\(^{2+}\)].

glycolysis; pentose phosphate pathway; adenosine 5'-triphosphate; carbon-13 nuclear magnetic resonance; metabolic regulation; A-23187

SEVERAL STUDIES INDICATE that plasma free ionized magnesium can fall during severe illness and after cardiac surgery (1, 22, 23) and that clinical administration of MgSO\(_4\) improves patient outcome (3, 10). The intracellular magnesium concentration ([Mg\(^{2+}\)]i) of some cell types, such as lymphocytes (2) and smooth muscle cells (28), is sensitive to the extracellular magnesium concentration, and red blood cell [Mg\(^{2+}\)]i can fall after prolonged magnesium deficiency (21). Metabolic fluxes may be altered in those pathways that experience regulation via magnesium concentration, especially those for which MgATP or MgADP is a substrate. Even though the magnesium requirement for some of the individual purified enzymes of glycolysis has been reported (6, 17, 20), there are few studies of this requirement in the intact pathways found in living tissue. It has become clear that metabolic regulation experienced by a cell in vivo is a distributed phenomenon, which is sensitive to the fluxes through coupled pathways in addition to multiple metabolite effectors. The available MgATP or MgADP, for instance, can alter the rate of regulation by the MgATP or MgADP (4). Therefore, it is a poor assumption that the flux through an intact metabolic pathway will respond as though it were a solution of purified enzymes to changes in the concentration of a single effector. The erythrocyte provides a simple model system in which to study the effects of magnesium on an entire metabolic pathway in the absence of mitochondria or protein synthesis.

A second goal is to determine whether the measured effective concentration range of the regulating molecule falls within the range found in the intact cell. Recent findings from \(^{13}\)C- and \(^{31}\)P-NMR kinetic studies of human erythrocytes consuming [2-\(^{13}\)C]glucose in the presence of variable magnesium concentration showed that the [Mg\(^{2+}\)]i at half-maximal velocity ([Mg\(^{2+}\)]i,0.5) for the entire glycolytic pathway is 0.03 mM (14). [Mg\(^{2+}\)]i in these cells is between 0.2 mM in oxygenated and 0.7 mM in deoxygenated cells (9, 18), and therefore [Mg\(^{2+}\)]i is unlikely to be regulatory for glycolysis under normal, unstressed conditions. These experiments employed the divalent ionophore A-23187 to allow equilibration between intracellular and extracellular Mg\(^{2+}\), a \(^{31}\)P-NMR assay of ATP-bound Mg\(^{2+}\) to determine [Mg\(^{2+}\)]i, and \(^{13}\)C-NMR to monitor metabolism of the \(^{13}\)C-enriched glucose substrate (9, 14, 25).

In these earlier experiments the pentose phosphate pathway activity (PPP) and rate of ADP phosphorylation were both too low to accurately measure their magnesium dependence. The PPP is important in erythrocytes to provide the NADPH used for glutathione reduction, which is in turn used to repair oxidized cellular components (26, 29). This may be especially important during inflammation, which is associated with increased release of reactive oxygen species and nitric oxide (12, 24). Therefore, the PPP is likely to be activated under the same conditions in which plasma magnesium is reduced. In the current study, the PPP was maximally activated with the redox dye methylene blue. This dye oxidizes NADPH to produce NADP\(^{+}\), the substrate for the rate-limiting enzyme glucose-6-phosphate dehydrogenase (G-6-PDH) (3, 22). The putative sites for magnesium regulation are 6-phosphogluconate dehydrogenase and transketolase, which contains a tightly bound thiamin pyrophosphate and requires Mg\(^{2+}\) as a cofactor (11, 27).

ADP phosphorylation and the synthesis of adenine nucleotides from exogenous adenine and [2-\(^{13}\)C]glucose were also activated in the presence of methylene blue, allowing us to measure the magnesium dependence of these fluxes. In the erythrocyte, the rate of ADP phosphorylation can be less than the rate of flux through the glycolytic pathway, because the 2,3-diphosphoglycerate (2,3-DPG) shunt bypasses the enzyme phosphoglycerate kinase (PGK) to produce 2,3-DPG rather than ATP. The rate of [2-\(^{13}\)C]2,3-DPG appearance can be directly measured in the \(^{13}\)C-NMR experiment, and subtraction of this rate from net flux through the glycolytic pathway yields PGK flux, which...
is equal to the net rate of ADP phosphorylation. Likewise, production of [1-13C]adenine nucleotide from [1-13C]ribose 5-phosphate (R-5-P) produced in the PPP can be directly measured in the 13C-NMR experiment.

These fluxes can be plotted against the measured [Mg2+]i to yield saturating curves that are reminiscent of simple Michaelis-Menten kinetics (velocity = [S] × Vmax/[S] + Km, where [S] is the substrate concentration, Vmax is the maximum velocity, and Km is the Michaelis-Menten constant). Clearly, the Michaelis-Menten formalism can be strictly applied only to well-controlled measurements of the initial reaction rates of single-substrate reactions catalyzed by purified enzymes. This is far from the case when the metabolic fluxes in an intact cell are measured, where alterations of one effector can result in changes in many substrate and product concentrations, as well as in energy-dependent processes such as membrane transport. Michaelis-Menten formalism, however, useful for describing the general characteristics of control by an effector. Therefore, much of the following data has been fit to this equation to yield an overall Vmax. Rather than a Km, an analogous value, the intracellular magnesium ion concentration at half-maximal velocity, [Mg2+]0.5, will be reported.

METHODS

Methods were essentially those found in Refs. 9, 14, and 25. Blood (5 ml) drawn from healthy volunteers who had given informed consent was centrifuged, and erythrocytes were washed with PBS and incubated at 3% hematocrit for 60 min at 37°C in a solution containing 110 mM NaCl, 5 mM KCl, 40 mM HEPES, 15 mM Na2HPO4, 5 mM adenine, 10 mM glucose, 6.6 µM methylene blue, pH 7.4, and MgCl2 between 0 and 1.6 mM. A-23187 (6 µM), a divalent ionophore, was included to allow equilibration of magnesium ion across the cell membrane, and 1 mM EGTA was included to bind residual calcium. This solution was found to promote maximal glucose use. Magnesium ion concentration in the buffer was determined by ion-sensitive electrode with an appropriately calibrated clinical analyzer (NOVA Biomedical, Waltham, MA). Cells were subsequently washed twice in the same buffer without glucose and resuspended at 50% hematocrit with 1 mM glucose, oxygenated in 100% O2 on ice, and placed in a 10-mm tube for the NMR experiment.

After an initial 20-min 31P-NMR spectrum (34°C, 60° pulses, 2-s relaxation delays, composite pulse decoupling), 10 mM [2-13C]glucose was added, and glucose, lactate, PO2, pH, and Mg2+ were measured with commercial analyzers from NOVA Biomedical. Fifteen 10-min 13C-NMR spectra were taken (same parameters), followed by a final two 31P spectra. The 13C-NMR spectra were used to calculate [Mg2+]i from the chemical shift difference between the α- and β-ATP peaks. The intensities of the following 13C-labeled metabolites were measured: [2-13C]glucose, [2-13C]lactate, [3-13C]lactate, [2-13C]2,3-DPG, [3-13C]2,3-DPG, and [1-13C]adenine nucleotide. Cells were frozen in liquid N2, stored at −50°C, and extracted with perchloric acid. 13C-NMR spectra of extracts were taken for determination of 13C in different molecules, and 1H-NMR spectra were taken for measurement of the enrichment with 13C at C-3 of lactate [1.3 parts per million (ppm) in 1H spectra] and C-1 in ribose of ATP (6.11 ppm). Enrichment was calculated as the ratio of the area under the 1H-13C doublet to the total combined area, including the 1H-13C central singlet (13). 2,3-DPG was measured in trichloroacetic acid extracts (Kit 665-PA, Sigma Chemical, St. Louis, MO). 13C-NMR data were analyzed as in Ref. 14. Because of the spectral broadening that occasionally occurred at late times in the experiments, [Mg2+]i was calculated from ATP resonances in the initial 31P-NMR spectrum (9, 14), and all reported rate data are calculated as the slope at 30 min of 13C-NMR peak areas plotted against time. All calculations are shown in the Appendix.

RESULTS

Glucose consumption was measured as the slope at 30 min of [2-13C]glucose NMR peak area plotted vs. time, and the results are plotted against the initial [Mg2+]i for each experiment in Fig. 1. Although these data measured in a living system show a great deal of scatter, it is clear that [Mg2+]i is participating in the regulation of glycolysis flux at the very low end of the physiological range of 0.2–0.7 mM. Overall Vmax and [Mg2+]0.5 for glucose use are both slightly higher than found in the absence of methylene blue (Ref. 14, Table 1).

Figure 2 shows PC, the fractional flux through the PPP, which falls exponentially as a function of [Mg2+]i. PPP activity is shown in Fig. 3. The dotted line is derived from the functions that represent the best fits in Figs. 1 and 2, and the circles are derived from the individual rates of glucose utilization. The dotted line therefore does not represent a best fit of the individual data points but does suggest a shape for the magnesium dependence of the PPP. The estimated [Mg2+]0.5 of 0.02 mM is well below the physiological range. However, it appears that the flux through the PPP is decreased in the physiological range between 0.2 and 0.7 mM [Mg2+]i. This is unlikely to be due to direct inhibition of this pathway by magnesium but may be a response to activation of an enzyme in the competing glycolytic pathway by magnesium in this range.
Table 1. Kinetic parameters for erythrocyte glucose metabolism

<table>
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<th>Control*</th>
<th>Methylene Blue†</th>
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<tr>
<td></td>
<td>[Mg²⁺]₀.₅, mM</td>
<td>V_max</td>
</tr>
<tr>
<td>Total glucose utilization</td>
<td>0.03</td>
<td>0.44</td>
</tr>
<tr>
<td>PPP</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>F-6-P—GAP</td>
<td>0.03</td>
<td>0.44</td>
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<tr>
<td>GAP—lactate</td>
<td>0.03</td>
<td>0.44</td>
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<tr>
<td>2,3-DPG shunt</td>
<td>0.02</td>
<td>0.35</td>
</tr>
<tr>
<td>PGK</td>
<td>0.08</td>
<td>0.27</td>
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All V_max units are μmol glucose equivalents·min⁻¹·g hemoglobin⁻¹. *Data from Ref. 13. †Current data.

Figure 4 shows the flux through the portion of the glycolytic pathway through phosphofructokinase (PFK), i.e., between fructose 6-phosphate (F-6-P) and glyceraldehyde 3-phosphate (GAP). The circles are calculated from the individual data points in Fig. 1 and the exponential curve in Fig. 2, and the dotted line is the least-squares fit to the Michaelis-Menten equation (Table 1). Flux through the span that contains glyceraldehyde 3-phosphate dehydrogenase (GAPDH), GAP—lactate, expressed in terms of micromole glucose equivalents per minute per gram hemoglobin, is shown in Fig. 5. The fractional fluxes through the 2,3-DPG shunt and the enzyme this shunt bypasses, PGK, were measured from the initial rates of the fitted time courses of 2,3-[3-¹³C]DPG and 2,3-[¹³C]lactate, and are also shown in Fig. 5 (14). The behavior of each of these fluxes was fit to the Michaelis-Menten equation, although this fit required an offset for [Mg²⁺] in the case of PGK. Flux through the enzymes of the 2,3-DPG shunt, 2,3-DPG mutase, and 2,3-DPG phosphatase had little or no dependence on [Mg²⁺], in the physiological range, and the derived kinetic constants are very similar to those obtained in the absence of methylene blue (Table 1). On the other hand, PGK had no apparent activity until [Mg²⁺] was above 0.13 mM, and then had a V_max of 0.19 μmol·min⁻¹·g hemoglobin⁻¹ and a [Mg²⁺]₀.₅ of between 0.2 and 0.3 mM. This indicates that net glycolytic ADP phosphorylation has a magnesium ion dependence that falls within the physiological range of concentration.

There was detectable ¹³C label in the C1 position of the ribose in adenine nucleotides, which resonates at 88 ppm in the in situ experiment and followed a saturating curve in time (Fig. 6). This indicates that at least part of the adenine nucleotide pool is in rapid turnover (synthesis and breakdown rates are equal),...

Fig. 2. PC (fractional pentose phosphate pathway (PPP) activity) as a function of [Mg²⁺]. PC was calculated from the ratio of 2,3-[3-¹³C]diphosphoglycerate (DPG) to 2,3-[¹³C]DPG in ¹³C-NMR spectra of perchloric acid extracts of erythrocytes. Data were fit to exponential shown, A烬巴+ C, where A = 0.364, B = 4.53, and C = 0.264. Symbols are the same as for Fig. 1.

Fig. 3. Flux through PPP as a function of [Mg²⁺]. Dotted line represents PPP calculated from fitted curves of Figs. 1 and 2 and Eq. A3. Also shown is PPP calculated from curve of Fig. 2 and individual data points of Fig. 1 (circles). No attempt was made to fit these data to simple function.

Fig. 4. Phosphofructokinase flux as function of [Mg²⁺]. Flux through glycolytic span between fructose 6-phosphate (F-6-P) and glyceraldehyde 3-phosphate (GAP) was calculated from Eq. A4. Circles were calculated as for Fig. 3 and dotted line is best fit to Michaelis-Menten equation, where V_max = 0.45 μmol·min⁻¹·g hemoglobin⁻¹ and [Mg²⁺]₀.₅ = 0.16 mM.
because at steady state, net synthesis would lead to a signal that is constantly increasing with time. The total $^{13}$C in the C-1 position at the end of the experiment was measured in extracts and is shown as a function of $[\text{Mg}^{2+}]_i$ in Fig. 7A. The fractional enrichment with $^{13}$C at this site was measured with $^1$H-NMR in extracts and was also a strong exponential function of $[\text{Mg}^{2+}]_i$.

$[18-^{13}$C]adenine nucleotide fractional enrichment equals $0.301 \pm 0.012 \exp(-26.57 \times [\text{Mg}^{2+}]_i)$ (data not shown).

The rate of formation of $[18-^{13}$C]adenine nucleotide could be calculated for seven experiments from the initial slope of the change in time of this signal and is plotted in Fig. 7B.

The fractional enrichment of the $[2^{13}$C]G-6-P pool was calculated for each experiment from Eq. A8 in the APPENDIX and is shown in Fig. 8A. G-6-P is the original substrate of the PPP, in which the ribose moiety of adenine nucleotide is made. The calculation does not account for dilution of the R-5-P pool with unlabeled material and therefore represents the maximal precursor fractional enrichment. This fractional enrichment could then be used to calculate the minimum rates of adenine nucleotide turnover at each $[\text{Mg}^{2+}]_i$, which are shown in Fig. 8B. Adenine nucleotide turnover was a strong function of $[\text{Mg}^{2+}]_i$, and was not saturated over the entire concentration range studied. The least-squares fit to a straight line yields the equation adenine nucleotide turnover equals $0.04 \times [\text{Mg}^{2+}] + 0.09$, with a correlation of $R^2$ equal to 0.494.

**DISCUSSION**

Gupta et al. (9) used $^{31}$P to measure the $[\text{Mg}^{2+}]$, in fully oxygenated human erythrocytes (0.25 mM) and in...
fully deoxygenated cells (0.67 mM). Under the current experimental conditions, the $[\text{Mg}^{2+}]_{\text{i}}$ in fully oxygenated cells was slightly lower, about 0.17 $\pm$ 0.02 mM ($n = 3$). The $[\text{Mg}^{2+}]_{0.5}$ for PPP was 0.02 mM, for PGK it was about 0.27 mM, and that for adenine nucleotide turnover was too high to determine with our experimental magnesium range. At normal physiological concentrations, then, $[\text{Mg}^{2+}]_{\text{i}}$ is important in the human erythrocyte for the regulation of PGK activity and adenine nucleotide synthesis from exogenous glucose and adenine, but is likely to be too high to directly affect PPP activity. This result compares well with the $K_m$ for Mg$^{2+}$ measured in purified PGK from human erythrocytes, which is 0.3 mM (20). In the erythrocyte, PGK can be bypassed by the 2,3-DPG shunt and can therefore experience much less flux than that of glucose through the entire pathway. However, this step is very important, because PGK flux is equal to the net rate of ADP phosphorylation (see Fig. 9). These cells should therefore be able to increase both ADP phosphorylation and ATP utilization purely as a function of $[\text{Mg}^{2+}]_{\text{i}}$ as they move from the arterial to the venous circulation.

It is not clear what the physiological role might be for this additional capacity for ATP generation in the anaerobic state. Glycolysis is increased in the anaerobic state (7, 25). This may be due to the fact that the free forms of 2,3-DPG, which inhibit several glycolytic enzymes including hexokinase (HK), PFK, PGK, and pyruvate kinase (20), bind tightly to deoxyhemoglobin and are decreased from 62% of total 2,3-DPG in the oxygenated state to 20% in the deoxygenated cell (18). Increased glycolysis and ATP formation may be important for ion pumping (16). Certainly the ATP-dependent Na$^+$/$\text{Mg}^{2+}$ exchange is activated both by artificial elevation of $[\text{Mg}^{2+}]$, and in the anaerobic cell, where $[\text{Mg}^{2+}]$ is also elevated (5, 9, 18).

The $V_{\text{max}}$ for total glucose utilization increased by 30% in the presence of methylene blue, and its $[\text{Mg}^{2+}]_{0.5}$ may also have been slightly altered (0.03 mM in control vs. 0.07 mM with methylene blue). The increase in $V_{\text{max}}$ was due entirely to increased PPP flux, because the calculated $V_{\text{max}}$ of the F-6-P $\rightarrow$ GAP span does not change on addition of a redox stress. This implies that we have been able to measure the true maximum activity of this portion of the glycolytic pathway. The apparent magnesium dependence of the F-6-P $\rightarrow$ GAP span ($[\text{Mg}^{2+}]_{0.5}$) is, however, elevated in the presence of methylene blue (Table 1). This could mean that the magnesium dependence of the regulatory enzymes in this portion of the glycolytic pathway were altered or that the rate control for glycolysis was no longer found in the F-6-P $\rightarrow$ GAP span. It could also mean that another factor besides

![Fig. 8. [2-13C]glucose 6-phosphate (G-6-P) enrichment and rate of adenine nucleotide turnover.](http://ajpendo.physiology.org/)

![Fig. 9. Pathways of glucose utilization in the erythrocyte.](http://ajpendo.physiology.org/)
[Mg$^{2+}$] becomes important for setting the flux rate. The last explanation is likely because of competition between PFK and activated G-6-PDH for the combined G-6-P and F-6-P substrate pool. G-6-P and G-6-P are thought to be in equilibrium. The $K_M$ of purified PFK for F-6-P is about 0.3–0.5 mM (17, 20). The normal concentration of F-6-P in human erythrocytes is 10–20 µM (8, 19), and it would likely be decreased by flux of G-6-P into the activated PPP. PPP flux should be far less dependent on G-6-P + F-6-P concentration, because the $K_M$ of G-6-PDH for G-6-P is 7.4 µM, well below the cellular concentration of about 40 µM (15, 19).

The PPP also has a much lower dependence on [Mg$^{2+}$] than the F-6-P–GAP span of glycolysis under our conditions ([Mg$^{2+}$]$_{0.5}$ ~ 0.02 mM). The fact that PC falls with increasing [Mg$^{2+}$], (Fig. 2) means that each individual glucose skeleton turns through the PPP cycle more times at lower [Mg$^{2+}$] than it does at higher [Mg$^{2+}$] when glucose uptake and PFK flux are elevated. This very low requirement of the PPP for Mg$^{2+}$ supports the work of Thorburn and Kuchel (26), who concluded using model systems that HK is rate limiting for PPP activity in oxidatively stressed human erythrocytes. We do not, however, concur with the literature value of $K_M$ for Mg$^{2+}$ of 1.0 mM for purified human erythrocyte HK, because the rate-limiting step has a [Mg$^{2+}$]$_{1.05}$ equal to 0.02 mM whether or not the PPP has been activated (6). Rather, our results imply that the PPP enzymes that require magnesium (presumably 6-phosphogluconate dehydrogenase and transketolase, Refs. 11 and 27) have [Mg$^{2+}$]$_{1.05}$ values that are much lower than 0.02 mM.

Competition for substrate between PFK and G-6-PDH would likely depress flux through PFK at low [Mg$^{2+}$] levels, because of the low [Mg$^{2+}$]$_{0.5}$ of the PPP and the low $K_M$ of G-6-PDH for G-6-P. PPP activation would therefore cause the observed increase in the measured apparent [Mg$^{2+}$]$_{1.05}$ for PFK. As [Mg$^{2+}$] is increased, the flux through the entire glycolytic pathway increases, perhaps due to activated PGK activity ([Mg$^{2+}$]$_{1.05}$ = 0.27 mM). This may contribute to the apparent decrease in flux through the PPP seen in the range of 0.15–0.6 mM [Mg$^{2+}$].

The rate of turnover of the adenine nucleotide pool continues to increase with [Mg$^{2+}$] well after glucose uptake and PPP have reached their plateau. The curve of adenine nucleotide turnover does not saturate with [Mg$^{2+}$], and therefore it is not possible to estimate values for $V_{max}$ or [Mg$^{2+}$]$_{0.5}$. There are few measurements because adenine nucleotide pool turnover was easily detected at high [Mg$^{2+}$], but the signal-to-noise ratio of the $^{13}$C peak at 88 ppm in the in situ spectra was often poor at low [Mg$^{2+}$] (see Fig. 7A). The adenine nucleotide turnover rate was calculated by assuming that the $^{13}$C enrichment of the 5-[1-$^{13}$C]phosphoribosyl-1-pyrophosphate (PRPP), the immediate precursor of newly synthesized AMP in erythrocytes, is the same as that of [2-$^{13}$C]G-6-P (shown in Fig. 8A) (20). This is a reasonable assumption at isotopic equilibrium, because the only point of label dilution between G-6-P and PRPP is at R-5-P and is due to exchange with the ribose moiety of adenine nucleotides. As shown in Fig. 6, the turnover of the PRPP pool is so rapid that it reached isotopic equilibrium well before the end of the experiment in all cases where the turnover rate could be measured. At the early time points, dilution of the [1-$^{13}$C]PRPP enrichment would be greater at the higher rates of adenine nucleotide pool turnover and would tend to make the slope of turnover vs. [Mg$^{2+}$], even steeper than shown in Fig. 8B.

The rapid turnover of adenine nucleotide pool in these experiments has an important impact on the calculation of PC. In the prior studies PC was calculated from the ratio of [3-$^{13}$C]lactate/[2-$^{13}$C]lactate (14, 25). In the current experiments, this ratio measured in blood extracts was always lower than the similar ratio, 2,3-[3-$^{13}$C]DPG/2,3-[2-$^{13}$C]DPG, even though 2,3-DPG and lactate have the same precursor pool. This is because of the turnover of the adenine nucleotides, which exchanges [1-$^{13}$C]R-5-P with the unlabeled ribose moiety and dilutes the enrichment of [3-$^{13}$C]lactate at early times. Label appearing as [2-$^{13}$C]lactate is not similarly diluted, because it does not pass through any unique, large metabolic pool. This problem of dilution only occurs when the $^{13}$C ratio of the growing lactate pool is used to calculate PC; the 2,3-DPG pool turns over constantly and quickly reaches a steady-state enrichment. Therefore, an end-product pool-like lactate that maintains a “history” of all tracer events during the experiment is not as good an indicator of PPP activity as a metabolically active pool like 2,3-DPG. The sum of label in C-1 of adenine nucleotides and [3-$^{13}$C]lactate could be divided by [2-$^{13}$C]lactate to obtain a number similar to the 2,3-DPG ratio (Eq. A2 in APPENDIX).

It is important to point out that although the reported fluxes through different parts of the glycolytic pathway could be measured somewhat independently, it is likely that the actual fluxes are very dependent on each other. For instance, the $V_{max}$ of ADP phosphorylation (PGK flux) may be dependent on the rate of adenine nucleotide turnover, which is dependent on adequate PPP activity. $V_{max}$ of PGK is, in fact, elevated by methylene blue (Table 1). Changes in [Mg$^{2+}$] per se may also alter fluxes independent of enzyme regulation. The most obvious manifestation of this is a potentially increased need for ATP (i.e., PGK flux) to fuel Na$^+$/Mg$^{2+}$ exchange (5).

In summary, the magnesium ion dependence of the pathways of glucose utilization was determined during activation of the PPP by methylene blue in human erythrocytes. The measured [Mg$^{2+}$]$_{0.5}$ values indicate that physiological [Mg$^{2+}$] is too high to play an important regulatory role for glucose uptake or PPP activity, although [Mg$^{2+}$] may be involved in the competition between G-6-PDH (the first committed step in the PPP) and PFK for substrate. Likewise, 2,3-DPG turnover is independent of [Mg$^{2+}$]. Net ADP phosphorylation, equal to PGK activity, has a [Mg$^{2+}$]$_{0.5}$ of 0.27 mM and is likely to be regulated by changes in [Mg$^{2+}$] in the physiological range. The rate of adenine nucleotide synthesis from exogenous adenine and glucose-derived R-5-P is
also highly dependent on [Mg^{2+}] in the physiological range of 0.2–0.7 mM. We have therefore determined that erythrocyte ATP production, both phosphorylation and adenine nucleotide synthesis, can be regulated by [Mg^{2+}] in its normal physiological range.

APPENDIX

The calculations are detailed in Refs. 14 and 25 and are presented briefly here with emphasis only on equations unique to the current study. Figure 9 shows the pathways of interest, where 1^{13}C-NMR visible metabolic pools, glucose, lactate, 2,3-DPG, and adenine nucleotides are shown in bold. Fluxes through six segments of the pathway could be estimated and these are numbered in Fig. 9.

Overall glucose utilization is measured as the change in the area of the [2-^{13}C]glucose peak over time (14, 25). PC is calculated and these are numbered in Fig. 9.

2,3-[3-^{13}C]DPG/[2-^{13}C]2,3-DPG = 2PC/(1 + 2PC) (A1)

In other studies (14, 25), PC was determined from the ratio in extracts of [3-^{13}C]lactate/[2-^{13}C]lactate. In the current studies we found that where both the 2,3-DPG and lactate ratios can be measured, the lactate ratio was always less than the 2,3-DPG ratio. This was because some of the carbon skeletons that enter the PPP are used to produce [1-^{13}C]R-5-P, which becomes incorporated into adenine nucleotides (resonance found at 88 ppm) under the conditions of high buffer adenine concentration and elevated PPP activity. This tends to reduce the total amount of [3-^{13}C]lactate produced during the entire experiment. 2,3-DPG is a metabolic pool that reaches a unique to the current study. Figure 9 shows the pathways of interest, where 1^{13}C-NMR visible metabolic pools, glucose, lactate, 2,3-DPG, and adenine nucleotides are shown in bold.

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Flux 1 = PPP = 3 × PC × glucose utilization (A3)

Two thirds of the material in the PPP reenters the glycolytic pathway as F-6-P and proceeds through PFK, defined as the span between F-6-P and GAP

Flux 2 = F-6-P → GAP flux = (1 − PC) × glucose utilization (A4)

On average, one molecule of GAP is produced for every six G-6-P that enter the PPP, and the flux of GAP through GAPDH can proceed to lactate through either PGK or the 2,3-DPG shunt

Flux 3 = GAP → lactate flux = (1 − PC/2)

× glucose utilization = flux 4 + flux 5 (A5)

The initial rates of [2-^{13}C]lactate and 2,3-[2-^{13}C]DPG appearance yield flux 4 and flux 5, respectively.

Flux 6, the rate of synthesis of the adenine nucleotide pool from PPP-derived R-5-P and exogenous adenine can be calculated from Eq. A6 if the fractional enrichment with ^{13}C of the R-5-P precursor, [2-^{13}C]G-6-P, is known

Flux 6 = adenine nucleotide synthesis

= d/dt([1-^{13}C]adenine nucleotide)/[^2-^{13}C]G-6-P enrichment (A6)

Appearance of ^{13}C in C-1 of the ribose moiety in adenine nucleotides (88 ppm) over time could be fit to Eq. A7. Most adenine and guanidine nucleotides resonate in this region, although the signal is predominantly adenine nucleotide. The shape of the curve indicates that the concentration of ^{13}C reaches a plateau when the metabolic pool is in isotopic equilibrium. This means that the rates of synthesis and degradation of the labeled product are equal. The initial rate of [1-^{13}C]adenine nucleotide appearance was taken as the derivative of Eq. A7 at 10 min after the signal at 88 ppm was observed

[1-^{13}C]adenine nucleotide = A(1 − e^{−t/τ}) + C (A7)

The fractional enrichment of [2-^{13}C]G-6-P can be calculated with Eq. A8

[2-^{13}C]G-6-P enrichment = 1/(1 + 2PC) (A8)

The following outlines the derivations of the expressions for [2-^{13}C]G-6-P fractional enrichment and the quantity PC. The calculation of PC is based on the assumption that the fractional enrichment of [1-^{13}C]G-6-P leaving the PPP is the same as the [2-^{13}C]G-6-P that was its precursor (25). The flow of G-6-P into the PPP is defined as 3PC × HK (Eq. A3), where HK is hexokinase flux. The average rate of F-6-P produced from the PPP is taken to be 2PC × HK, by the following stoichiometry

3[2-^{13}C]G-6-P + 6 NADP^{+} + 3H_{2}O = [1-^{13}C]F-6-P + 1[1,3^{13}C]F-6-P + 3CO_{2} + 1 GAP + 6 NADPH (A9)

The combined G-6-P + F-6-P sugar 6-phosphate (S-6-P) pool has two inputs in this model (Fig. 9), where all [2-^{13}C]S-6-P arrives through HK and all [1-^{13}C]S-6-P arrives from the PPP. If the enrichment of the precursor [2-^{13}C]glucose is 1.0, the enrichment at C-2 of the S-6-P pool, which gives rise to [2-^{13}C]lactate and 2,3-[2-^{13}C]DPG, is

[2-^{13}C]S-6-P/total S-6-P

= 1.0 HK/[HK(1 + 2PC)] = 1/(1 + 2PC) (A10)

The enrichment of S-6-P at C-1, which yields [3-^{13}C]lactate and 2,3-[3-^{13}C]DPG in the glycolytic pathway, can be calculated by assuming that at steady state the [1-^{13}C]F-6-P newly formed in the PPP has the same enrichment as the precursor [2-^{13}C]S-6-P pool

[1-^{13}C]S-6-P/total S-6-P = [2PC × HK]

× 1/(1 + 2PC)/HK(1 + 2PC) (A11)

= 2PC/(1 + 2PC)^{2}
The ratio of the two enrichments yields Eq. A1

\[
\frac{[1^{13}C]5-6-P/\text{[2}^{13}C\text{]}5-6-P}{2,3-[3^{13}C]DPG/2,3-[2^{13}C]DPG} = 2PC/(1 + 2PC)
\]

If the enrichment of F-6-P is less than that of its G-6-P precursor due to scrambling of label in the PPP, the ratio of Eq. A10 will underestimate PC and therefore the flux through PPP. If this were the case, we would expect to see a variety of splitting patterns in \(^{13}\text{C}\)-NMR spectra of the lactate and 2,3-DPG in extracts, as observed in Ref. 25. This splitting did not appear in the current study. If the adenine nucleotide pool is large and its turnover is very high, the enrichment of F-6-P will also be less than that of G-6-P, but only at early time points before the nucleotide pools have reached isotopic steady state.

Although every attempt was made to bring the system to metabolic steady state before measurements were made, this may not have been the case. If the adenine nucleotide pool expands during the experiment, \([1^{13}C]R-5-P\) would be lost as net adenine nucleotide synthesis, and Eq. A9 could be modified to

\[
(3 + x)[2^{13}C]G-6-P + (6 + 2x)\text{NADP}^+ + (3 + x)\text{H}_2\text{O} = [1^{13}C]F-6-P + [1,3^{13}C]F-6-P + x[1^{13}C]\text{adenine nucleotide}
\]

\[
+ (3 + x)\text{CO}_2 + 1\text{GAP} + (6 + 2x)\text{NADPH}
\]

In this case PC underestimates the fractional flux of GAP from PPP by the quantity \(x/3\). This would require a correction of PPP flux by about 0.02 μmol·min\(^{-1}\)·g hemoglobin\(^{-1}\) for each 1 mM increase of ATP. In the current study, any changes in ATP appeared to be very small (<1 mM).


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