Erythrocyte Membrane Polyphosphoinositide Metabolism and the Regulation of Calcium Binding*

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SUMMARY

The terminal phosphate of ATP is incorporated into erythrocyte membrane polyphosphoinositides at the rate of 1.5 nmoles per min per mg of protein in the presence of 10 mM Mg2+ and 0.5 mM ATP. The amount of membrane polyphosphoinositide monophosphate can be doubled in 30 min. Phosphorylation proceeds until the entire supply of membrane phosphatidylinositol is exhausted. Inhibition of incorporation by Ca2+ depends on the concentration of Mg2+ in the medium. Incorporation is also lower in the presence of detergent, although it may be restored to normal by the addition of exogenous phosphatidylinositol. An Arrhenius plot of reaction rate had two linear portions with a transition at 17°C.

As the amount of polyphosphoinositide in the membrane is increased, the amount of calcium bound is elevated. The lowest detectable binding site on the membrane (Kd, 4 x 10¹⁰ liters per mole) appears to be affected. Calcium bound at this site increases in a 1:1 molar ratio with increased polyphosphoinositide monophosphate. Phosphorylation of membrane protein is too low to account for the increase in calcium binding. Membranes with elevated polyphosphoinositide levels also have much higher Ca2+-ATPase activity than membranes with normal levels, although (Na+ + K+)-ATPase activity is not increased. The data provide the first direct evidence that the higher inositides may be involved in the regulation of intracellular calcium levels.

The polyphosphoinositides, diphosphoinositide and triphosphoinositide, are now known to be present in a wide variety of mammalian tissues (1-6), apparently localized in plasma membranes (7). Because of their rapid turnover in brain (8) and kidney (9) and the strongly ionic nature of their polar head groups, it has been suggested that they may be involved in the active transport of cations (8, 10). In the past, direct studies on the metabolism and function of DPI and TPI have largely been restricted to brain and kidney. In these tissues, however, interpretation of results is hampered by the complexity of the systems and the high activity of enzymes involved in polyphosphoinositide breakdown (8, 9). Recently Schneider and Kirschner (11) and Peterson and Kirschner (12) have reported remarkably high polyphosphoinositide levels in the swine erythrocyte and active incorporation of phosphate into membrane DPI and TPI. However, they were unable to find any correlation between phosphoinositide metabolism and Na+ or K+ transport. Both DPI and TPI have high affinities for divalent cations (13) and the indirect evidence of Harrison and Long (14) has suggested their involvement in the binding of calcium by the human erythrocyte membrane. We wished to further characterize erythrocyte phosphoinositide metabolism and investigate its possible relationship to calcium binding and calcium transport.

EXPERIMENTAL PROCEDURE

Materials

Swine blood was heparinized (20 units per ml) and normally used within 1 day of collection. Reagents for [γ-32P]ATP synthesis (15) were obtained from Sigma. [32P]Orthophosphate was obtained from Atomic Energy of Canada, Ltd., 45CaCl₂ (approximately 1.5 Ci per mmole) from Amersham-Searle Corporation. All other materials used were reagent grade from commercial sources. Distilled water was passed through a mixed bed ion exchange system before use.

Methods

Analytical Techniques—Lipids were extracted using the acidified chloroform methanol procedure of Welle and Littme (16). The two-dimensional thin layer system of Turner and Rouser (17) was used to quantitate the various lipid classes. Thin layer chromatography on oxalate-impregnated silicagel (18) was routinely used to isolate DPI and TPI. Lipid phosphorus was determined by the method of Bartlett (19), using perchloric acid for digestion. Protein was determined by the procedure of Lowry et al. (20).

Radioactivity Measurement—Aqueous solutions containing ⁴⁶Ca or ⁴⁵Ca were counted after adding 10 volumes of xylene containing 6 g per liter of diphenyloxazole, 0.12 g per liter of phenyloxazolyl-phenyloxazolylphenyloxazolylphenyloxazol, mixed 2:1 (v/v) with Triton X-100.

The abbreviations used are: DPI, diphosphoinositide; TPI, triphosphoinositide; PI, phosphatidylinositol.

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(Rohm and Haas). Lipid $^{32}$P was measured in toluene containing 5 g per liter of diphenyloxazole and 0.3 g per liter of phenylazophenylazonaphenal. Efficiency on a Nuclear Chicago MKII liquid scintillation counter was approximately 65% in each case. When low concentrations of $^{45}$Ca were measured, additional nonradioactive CaCl$_2$ was added to reduce wall effects.

**Enzyme Assays**—Unless otherwise noted, DPI and TPI synthesis was measured at 37°C using these incubation conditions: 5 mM $[\gamma-^{32}P]$ATP, 40 mM magnesium acetate, 50 mM Tris-HCl, pH 7.4, and 1 to 2 mg per ml of erythrocyte membrane ("ghost") protein. The reaction was terminated by adding 8 volumes of calcium was used for samples from tubes containing $[\gamma-^{32}P]$ATP.

$[Mg^{2+}]$ATPase was determined using the following conditions: 2 mM MgCl$_2$, 2 mM ATP, 50 mM NaCl, 5 mM KCl, 0.2 mM ouabain (Sigma), 50 mM Tris-HCl, pH 7.4, 0.5 mM disodium EDTA, and 1 to 2 mg of ghost protein per ml. $[Ca^{2+}]$ATPase was estimated after substituting 0.2 mM CaCl$_2$ for EDTA in the above reaction mixture.

Inorganic phosphate release was measured by the method of Chan et al. (22).

**Preparation of Erythrocyte Ghosts**—Membranes were prepared by the methods of Schneider and Kirschner (11) and were washed two to three times with 30 volumes of 20 mM Tris-HCl, pH 7.4, to remove EDTA. The white ghosts were resuspended in this medium at a concentration of 5 to 10 mg per ml and were frozen and thawed at least once to ensure breakage.

**Preparation of Phosphatidylinositol**—PI was isolated from fresh ox brain by a modification of the method of Long and Owens (23). Cellulose powder was omitted from the silicic acid column. Thin layer chromatography (24) showed that the preparation was free of contaminating phospholipids. The lipid was dried on the walls of a test tube under a stream of nitrogen and rapidly suspended in 50 mM Tris-HCl, pH 7.4, by swirling on a Vortex. The resulting emulsion was used immediately.

**Measurement of Calcium Binding during Phosphorylation of Membrane**—Ghosts (2 mg of protein per ml) were incubated in 10 mM magnesium acetate, 5 mM ATP or $[\gamma-^{32}P]$ATP, and 50 mM Tris-HCl, pH 7.4, for 2½ to 30 min. One-milliliter aliquots were removed at each time interval and rapidly mixed with 10 ml of ice-cold 1.0 mM $^{45}$CaCl$_2$, 20 mM Tris-HCl, pH 7.4. Unlabeled calcium was used for samples from tubes containing $[\gamma-^{32}P]$ATP. After centrifugation at 20,000 X g for 10 min, the ghosts were resuspended in 10 ml of 20 mM Tris-HCl and recentrifuged. The pellets containing $^{45}$Ca were suspended in 20 mM Tris-HCl and aliquots were counted to determine the amount of calcium bound. Lipids from pellets containing $^{32}$P were extracted as described above. Following separation into two phases, the total lower phase and interfacial protein radioactivity was determined. Levels of DPI and TPI in the lower phase were also estimated chemically after thin layer chromatography.

**Calcium Binding**—Membranes were incubated for 40 min under the conditions described for DPI and TPI synthesis, but in the presence and absence of ATP. Subsequent operations were carried out at 0°C. The membranes were washed twice with 30 volumes of 17 mM NaCl containing 1.44 mM Tris-HCl (pH 7.4) and 1.0 mM disodium EDTA to remove Mg$^{2+}$ and three times with 20 volumes of 20 mM Tris-HCl, pH 7.4, to remove EDTA. The ghosts were resuspended (5 to 10 mg of protein per ml) in 20 mM Tris-HCl and frozen before use. The hematocrit of the ghost suspension was 25%. To measure calcium binding, 0.2-ml portions were rapidly mixed with 2 ml of 20 mM Tris-HCl, pH 7.4, containing $10^{-4}$ to $10^{-3}$ m $^{45}$CaCl$_2$. Following centrifugation at 10,000 X g for 10 min, a 1-ml aliquot of the supernatant was taken for radioactivity measurement. The amount of calcium bound was calculated from the difference in radioactivity before and after ghost addition.

**RESULTS**

**Incorporation of $[\gamma-^{32}P]$ATP into Polyphosphoinositides**—The terminal phosphate of radioactive ATP was actively incorporated into ghost DPI and TPI (Fig. 1) in agreement with the observations of Schneider and Kirschner (11). Under our conditions, the incorporation rate was constant for 3 to 5 min at 1.5 to 2.0 nmole of phosphate per min per mg of protein. This rate is higher than that reported earlier (11), probably reflecting different incubation conditions and a less cumbersome method of phosphoinositide isolation. Careful examination indicated that no membrane lipids were labeled under these conditions and for this reason, total incorporation of phosphate into polyphosphoinositide was often measured simply by counting an aliquot of the total lipid fraction.

**Cofactor Requirements for Phosphate Incorporation**—The phosphorylating system required 10 mM Mg$^{2+}$ for optimal activity as reported by Schneider and Kirschner (11). In the presence of high Mg$^{2+}$ concentrations, phosphorylation was optimal with 1.0 mM ATP (Fig. 2). Calcium inhibited half-maximally at 0.5

![Fig. 1. Incorporation of $[\gamma-^{32}P]$ATP into polyphosphoinositides.](http://www.jbc.org/)

![Fig. 2. Effect of ATP on incorporation of $^{32}$P into polyphosphoinositides.](http://www.jbc.org/)
Fig. 3. Effect of Ca\(^{2+}\) on polyphosphoinositide synthesis. Incubation mixtures contained either 40 mM or 2 mM Mg\(^{2+}\) and varying amounts of Ca\(^{2+}\). Incubation time was 5 min.

**TABLE I**

Phospholipid composition of erythrocyte ghosts

Ghosts were incubated as described under "Experimental Procedure" for 30 min in the presence or absence of 40 mM magnesium acetate. The isolation and determination of the various phospholipids is also described under "Experimental Procedure." Phosphatidylinositol loss on incubation with Mg\(^{2+}\) was 22.1 nmoles per mg of protein; polyphosphoinositide increase on incubation with Mg\(^{2+}\) was 19.1 nmoles per mg of protein.

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Without Mg(^{2+})</th>
<th>With Mg(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPI and TPI</td>
<td>4.9 ± 0.7</td>
<td>9.8</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>3.8 ± 0.2</td>
<td>4.0</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>19.1 ± 0.3</td>
<td>19.0</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>26.7 ± 0.5</td>
<td>26.8</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>22.0 ± 1.1</td>
<td>22.7</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>22.0 ± 0.7</td>
<td>21.7</td>
</tr>
</tbody>
</table>

\(^a\) Mean of four experiments ± standard error of the mean.

\(^b\) Mean of two experiments.

\(^c\) Not detectable.

max with 20 mM Mg\(^{2+}\) and at lower concentrations when the level of Mg\(^{2+}\) was decreased (Fig. 3).

Phospholipid Distribution in Erythrocyte Membranes—The distribution of major phospholipid classes in ghosts incubated in the presence and absence of ATP is presented in Table I. The values for ghosts incubated in the absence of ATP are generally in agreement with those reported by Nelson (25) for whole cells. No lysolcinithin was detected, and although a trace of phosphatic acid was noted, it was below our measurement capabilities. It can be seen from the table that incubation in the presence of ATP resulted in the complete disappearance of PI and a corresponding increase in the levels of DPI and TPI.

Effect of Detergent and Exogenous Diphasphoinositide—When enzymes involved in polyphosphoinositide metabolism are assayed using exogenous PI, detergents are often added as activators (e.g., Reference 7). In our system, with membrane PI as substrate, 1.5% Cutscum lowered enzyme activity (Fig. 4). Activity was nearly completely restored by adding exogenous PI to the reaction mixture (Fig. 5), whereas the enzyme was inhibited by PI in the absence of detergent.

Effect of Temperature—When phosphorylation was measured over the range 0–37° and the results were expressed as an Arrhenius plot a curve with two linear portions and a break near 17° was obtained (Fig. 6).

Polyphosphoinositide Breakdown in Whole Cell Hemolysates—Frozen and thawed cells were able to catabolize DPI and TPI, with DPI apparently the preferred substrate (Fig. 7). Erythrocyte ghosts also showed breakdown ability, although this varied widely from preparation to preparation and seemed to depend on the presence of small amounts of EDTA. No transitory increases in DPI levels were observed in any experiments in which TPI breakdown was measured.

Polyphosphoinositides and Calcium Binding—The amount of calcium bound to ghost membranes increases as incorporation of phosphate into DPI and TPI continues (Fig. 8). The molar ratio of increased calcium to increased phosphoinositide monophosphate is approximately 0.8:1.0 over the time of the experi-
Fig. 6. Effect of temperature on $^{32}$P incorporation. The ghosts and the rest of the reaction mixture were equilibrated separately at each temperature before mixing. Incubation time was 5 min.

Fig. 7. Breakdown of polyphosphoinositides in a whole cell hemolysate. Intact cells were previously labeled by incubation for 3 hours with $[^{32}P]$orthophosphate and adenosine (1 mg per ml) in phosphate-free Ringer solution. The cells were washed, then frozen and thawed twice in an equal volume of a solution containing 140 mM KCl, 10 mM NaCl, 2.5 mM MgCl$_2$, 0.5 mM CaCl$_2$, and 5 mM ATP (pH 7.4).

Fig. 8. Calcium binding and the phosphorylation of ghost polyphosphoinositol and protein. See "Experimental Procedure" for details of the method. The actual amount of TPI monoesterified phosphate increased from 32 to 54.5 nmoles per mg of protein. DPI monoesterified phosphate increased from 4.9 to 10.0 nmoles per mg of protein.

Fig. 9. Scatchard plot of calcium binding to erythrocyte membranes. Ghosts were previously incubated and calcium binding was determined as described under "Experimental Procedure." The lowest detectable binding site ($K_{app} \approx 4 \times 10^4$ liters per mole), appears to be the one affected by increasing membrane polyphosphoinositides. The increment in calcium bound at this site corresponds to the increment in phosphoinositol monophosphate following incubation with ATP (Table II).

Phosphoinositides and Ca$^{2+}$-ATPase—Increasing the polyphosphoinositide levels in ghosts results in a slight increase in Mg$^{2+}$-ATPase activity and a dramatic increase in Ca$^{2+}$-ATPase activity (Table III). Magnesium-activated (Na$^+$ + K$^+$)-ATPase is not affected, in agreement with the findings of Peterson and Kirschner (12). The action of polyphosphoinositides on Ca$^{2+}$-ATPase may be to increase the sensitivity of the enzyme's active site to calcium, or to increase the effective concentration
TABLE II
Calcium binding and polyphosphoinositide increases in erythrocyte membranes

The data are from Fig. 9. Enriched membranes contained 18 nmoles more polyphosphoinositide monophosphate per mg of protein and bound 20 nmoles more calcium per mg of protein at the site corresponding to $K_0$.

<table>
<thead>
<tr>
<th>Stability constant</th>
<th>Control membranes (lower curve)</th>
<th>Polyphosphoinositide-enriched membranes (upper curve)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_0$</td>
<td>$4 \times 10^4$</td>
<td>$4 \times 10^4$</td>
</tr>
<tr>
<td>$K_1$</td>
<td>$5 \times 10^2$</td>
<td>$5 \times 10^2$</td>
</tr>
</tbody>
</table>

TABLE III
Effect of increased membrane polyphosphoinositide on ATPase activities

One enzyme unit releases 1 n mole of P$_i$ per min per mg of protein. Assay methods are described under "Experimental Procedure." Both Ca$^{2+}$-ATPase and (Na$^+$ + K$^+$)-ATPase activities are corrected for Mg$^{2+}$-ATPase activity.

<table>
<thead>
<tr>
<th>ATPase</th>
<th>Polyphosphoinositide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>2.1</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>7.8</td>
</tr>
<tr>
<td>Na$^+$ + K$^+$</td>
<td>4.2</td>
</tr>
</tbody>
</table>

FIG. 10. Effect of polyphosphoinositide increases on the calcium dependence of Ca$^{2+}$-ATPase. Membranes were previously incubated with (X—X) and without (O—O) ATP. Following several washes with 20 mM Tris-HCl, pH 7.4, Ca$^{2+}$-ATPase was estimated as described under "Experimental Procedure."

of membrane calcium. If the former is the case, it might be expected that ghosts with increased polyphosphoinositide levels might reach maximal Ca$^{2+}$-ATPase activity at lower medium calcium concentrations. The data in Fig. 10 indicate that this is not the case.

DISCUSSION

The specific activity reported here for the erythrocyte membrane system phosphorylating PI compares favorably to the values reported by Schneider and Kirschnier (11), when their data are corrected to optimal Mg$^{2+}$ concentrations. The specific activity is much higher than that reported by Harwood and Haw-thorne (7), probably because their assay system contained 5 mM PI in the absence of detergent, leading to a considerable reduction in phosphate incorporation (Fig. 5). In agreement with Schneider and Kirschnier (11), we could find no other labeled phospholipids in the membranes after incubation with [γ-32P]ATP. Thus the swine red cells differ from human erythrocytes which exhibit diglyceride kinase activity (3, 28). As the swine erythrocyte cannot utilize diglyceride to produce PI, the phosphodiesterase hydrolyzing DPI and TPI to diglyceride and inositol phosphates is probably not present. Its activity would lead to the irreversible loss of inositol phosphates from the erythrocyte. Thus it is more likely that the breakdown observed (Fig. 7) is caused by TPI phosphomonoesterase

$\text{TPI + H}_2\text{O} \rightarrow \text{DPI + P}_1$

$\text{DPI + H}_2\text{O} \rightarrow \text{PI + P}_1$

This phosphomonoesterase has been reported to be at least partially soluble (29), which may account for the variable activities we observed in erythrocyte membranes.

The inhibitory effect of Cutsicum on phosphorylation (Figs. 4 and 2) is not unexpected, since the effective concentrations of enzyme and substrate would be much higher in the intact membrane than after solubilization by this detergent.

Exogenous PI stimulates phosphorylation in the presence of detergent, although the activity observed in native membranes is never surpassed. The inhibition by PI in the absence of detergent is difficult to explain.

An Arrhenius plot of reaction rate indicates that the phosphorylating system undergoes a phase transition at 17° (Fig. 6). Phase transitions near this temperature have been reported in bacteria (30, 31) and mitochondria (32). Buckley* has observed a transition in the rate of lecithin exchange with plasma and total ATPase activity near 17° in rat erythrocytes. These transitions probably reflect a change in the physical state of the membrane phospholipids (33).

Harrison and Long (14) noted that intracellular calcium levels in human erythrocytes were comparable with cellular levels of DPI and TPI and Long and Mount (27) attributed the calcium-binding site of highest affinity to polyphosphoinositides. The data presented in Figs. 8 and 9 confirm that membrane calcium binding is directly related to the levels of membrane DPI and TPI. The molar ratio of Ca$^{2+}$ to polyphosphoinositide monophosphate is approximately 1:1. Thus phosphorylation of membrane PI may provide a mechanism for the regulation of intracellular Ca$^{2+}$ levels. The inhibition of polyphosphoinositide synthesis by Ca$^{2+}$ (Fig. 3) may not be a factor at the very low calcium levels (less than 20 μM) reported for erythrocytes (14).

The striking increase in Ca$^{2+}$-ATPase, which was always observed when membrane DPI and TPI concentrations rose (Table III), may be secondary to the increase in membrane calcium levels. We attempted to measure Ca$^{2+}$ transport as well as Na$^+$ and K$^+$ transport in resealed cells in order to observe whether or not the phosphoinositides were directly involved. However, all such attempts have been unsuccessful so far, due to the equilibration of polyphosphoinositides levels during the time required for resealing the cells after addition of the cations and ATP.

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