Phosphoinositide Metabolism and the Morphology of Human Erythrocytes

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ABSTRACT ATP-depleted human erythrocytes lose their smooth discoid shape and adopt a spiny, crenated form. This shape change coincides with the conversion of phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol and phosphatidic acid to diacylglycerol. Both crenation and lipid dephosphorylation are accelerated by iodoacetamide, and both are reversed by nutrient supplementation. The observed changes in lipid populations should shrink the membrane inner monolayer by 0.6%, consistent with estimates of bilayer imbalance in crenated cells. These observations suggest that metabolic crenation arises from a loss of inner monolayer area secondary to the degradation of phosphatidylinositol-4,5-bisphosphate and phosphatidic acid. A related process, crenation after Ca²⁺ loading, appears to arise from a loss of inositides by a different pathway.

The shape of human erythrocytes is under metabolic control; after cells exhaust their ATP they become spiculate spheres. This shape change (called crenation or echinocytosis) eventually becomes irreversible, but for many hours crenated cells can revert to normal discoid shape if ATP is restored by appropriate nutrients (1).

Certain amphipathic molecules induce shape changes similar to metabolic crenation in ATP-replete cells, whereas other amphipaths produce what appears to be the reverse process, stomatocytosis. To explain the morphological effects of amphipaths, Sheetz and Singer (2) proposed the bilayer couple hypothesis, which attributes cell shape to the relative areas of the plasma membrane monolayers. According to this model, cationic amphipaths capable of rapid trans-bilayer flip-flop (e.g., chlorpromazine) would be expected to bind preferentially to the acidic phospholipids of the inner leaflet, expanding its area and causing the cell to cup. Anionic amphipaths (e.g., barbiturates) and molecules incapable of fast flip-flop (e.g., methochlorpromazine) should accumulate in the outer leaflet, expanding it and thereby producing the spiny shape of echinocytes. Although serious objections to this model have been raised (3) and countered (4, 5), it is indisputable that the bilayer couple hypothesis successfully predicts the morphological effects of a wide variety of drugs. However, it is not apparent how metabolic depletion might induce bilayer imbalance.

Other workers have suggested that erythrocyte shape might be controlled by the membrane protein cytoskeleton acting as a scaffolding upon which the rest of the membrane is built. Circumstantial support for this idea arose from studies of Triton shells, the proteinaceous remains of detergent-treated ghosts. Early reports (6–8) indicated that these cytoskeletal shells retain the shape of the cell from which they are derived (discocytic, elliptocytic, irreversibly sickled), suggesting that they possess a long-term morphological memory.

Subsequent work by Sheetz, Birchmeier, and Singer (9, 10) provided further support for the idea that the cytoskeleton controls red cell shape, and suggested how ATP depletion leads to crenation. They proposed that metabolic control of the cytoskeleton is exerted through the phosphorylation and dephosphorylation of the β-subunit of the protein spectrin. This assertion was supported by Pinder et al. (11) who found that the viscosity of crude mixtures of spectrin and other cytoskeletal components varies with the degree of spectrin phosphorylation. ATP dephosphorylation would lead to spectrin dephosphorylation, which was thought to rearrange the cytoskeleton and contract the inner monolayer (10). The resulting bilayer imbalance would force the cell to crenate.

This hypothesis was largely confuted by three observations. First, α-adrenergic stimulation increases spectrin phosphorylation without detectable morphological consequences (12). Second, much of the spectrin kinase activity of ghosts can be extracted or inhibited without altering their capacity to become discoid (13, 14). Finally, if spectrin dephosphorylation were to cause metabolic crenation, it should either precede the shape change or occur concurrently. Anderson and Tyler (15) showed that in intact cells, spectrin dephosphorylation lags behind metabolic crenation. These observations all show...
that there is no simple relationship between spectrin phosphorylation and cell shape. Indeed, it has yet to be demonstrated that spectrin phosphorylation has any functional consequences (15, 16).

Recently Lange and co-workers (17, 18) have found that Triton shells do not always echo the shape of the parent cell. These workers concluded that the cytoskeleton probably does not control cell shape. Rather, previously noted similarities between cells and Triton shells could reflect the tendency of the cytoskeletal reticulum to conform (with short-term elasticity and long-term plasticity) to the shape dictated by the lipid bilayer.

In 1975, Allan and Michell (19) found elevated levels of diacylglycerol in echinocytes formed by either metabolic depletion or calcium loading. They proposed that this lipid triggers creation, and that its fusogenic activity induces the membrane budding (microvesiculation) observed subsequent to creation. Later, they and their co-workers showed that calcium loading activates a phospholipase C, which degrades phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate. In ATP-replete cells, the resulting diacylglycerol is quickly phosphorylated to yield phosphatidic acid, while creation proceeds (20). This observation negated the hypothesis that diacylglycerol accumulation per se causes creation, but left open the possibility that related processes might be involved in morphology changes.

In this report, we examine inositol metabolism during both metabolic creation and its reversal. Our findings support the conclusion that metabolic creation is caused by lipid bilayer imbalance secondary to the loss of phosphatidylinositol-4,5-bisphosphate and phosphatidic acid from the inner monolayer of the cell membrane.

MATERIALS AND METHODS

Human erythrocytes were obtained by venipuncture, pelleted by centrifugation, and washed three times with 4 vol of 150 mM NaCl and once with 138 mM NaCl, 5 mM KCl, 6.1 mM Na2HPO4, 1.4 mM NaH2PO4, 1 mM MgSO4, 5 mM glucose, pH 7.4 (NaCl/Pi). Cells were maintained within 6 h of being drawn. All of the incubations described below were carried out at 37°C, hematocrit 20%, in capped plastic tubes.

Prelabeling Cells with 32P

Erythrocytes were labeled with 32P by incubation for 18–30 h with NaCl/Pi supplemented with 10 mM inosine, 1 mM adenine, and an additional 5 mM glucose (supplemented NaCl/Pi). The suspension medium contained antibiotics to retard bacterial growth (penicillin G, 100 µg/ml, plus either streptomycin, 100 µg/ml, or tobramycin, 40 µg/ml) and ~25 µCi/ml 32P2H3PO4 (ICN Nutritional Biochemicals, Cleveland, OH, carrier-free). In one experiment, supplemented NaCl/Pi was replaced with NaCl/Pi to which 10 mM glucose was added every 7–8 h. This protocol maintained ATP levels less than 10% of control values, and 2 of the results of the experiment were otherwise similar to the results of a duplicate experiment using supplemented NaCl/Pi. Incubations of this length allowed equilibration of ATP, phosphatidylinositol-4-phosphate, and phosphatidylinositol-4,5-bisphosphate (but not phosphatidic acid) with the radiolabeled phosphate.

Metabolic Depletion and Repletion

Metabolic depletion was initiated by one of two procedures. (a) Slow ATP depletion: 32P-labeled cells were pelleted, washed once in 4 vol of 150 mM NaCl, and resuspended in 140 mM NaCl, 10 mM HEPES, pH 7.4, plus penicillin G (100 µg/ml) and tobramycin (40 µg/ml). This treatment induces ATP depletion and creation with half-times of ~12 and 34 h, respectively. (b) Rapid ATP depletion: 32P-labeled cells were pelleted, washed three times in 4 vol of 150 mM NaCl, and resuspended in 140 mM NaCl, 10 mM HEPES, 10 mM inosine, 6 mM iodoacetamide, pH 7.4. In this medium, cells deplete their ATP within 1 h, and creation follows rapidly (21).

The reversal of metabolic creation was examined in echinocytes produced by procedure a. The echinocytes were pelleted, washed once in NaCl/Pi, and resuspended in supplemented NaCl/[32P]Pi, a portion of which had been used in the prelabeling phase of the experiment.

Assay Procedures

MORPHOLOGY: Erythrocytes were diluted to hematocrit 5 and fixed in 150 mM NaCl containing 0.5% glutaraldehyde (Sigma Chemical Co., St. Louis, MO) for 20 min, which produces the "glass effect" (22). Echinocytes were examined by bright field microscopy with oblique illumination, a procedure that makes the cell image appear three dimensional (23). Some cell samples were placed on Nuclepore filters (Pleasanton, CA), freeze-dried, sputter-coated, and examined by scanning electron microscopy. Echinocytes were assigned a morphology score of 1–5 based on Bassis's nomenclature for echinocytes and stomatocytes (22). Discocytes were assigned a score of 0, and stage I stomatocytes, observed in a few fields, were assigned a score of 1. The average score for a field of 100 cells was called its morphological index (MI).

ATP: Frozen aliquots of cell suspension (50 µl) were diluted in 2.45 ml of 150 mM NaCl, boiled for 15 min, and centrifuged to clarify. The supernatant (diluted 1:10 or undiluted) was assayed for ATP by the luciferin-luciferase (Calbiochem-Behring Corp., San Diego, CA) method (24).

LIPOSIDES: Frozen aliquots of cell suspension (100 µl) were lyzed in 1 ml cold 10 mM Tris/2 mM EDTA, pH 7.5, to prevent hydrolysis of radiolabeled lipids (25, 26). Samples were centrifuged for 5 min at 8,800 g, and the pellets were washed once in Tris/EDTA. The entire ghost pellet was extracted with 100:50:1 (vol/vol) methanol/chloroform/concentrated HCI (1.8 ml) followed by water (0.6 ml) and chloroform (0.6 ml). The organic phase was separated and the aqueous phase extracted again with 1.2 ml chloroform. The organic phases were combined, dried under a stream of nitrogen, and dissolved in two 10-µl portions of chloroform. Lipids were separated by thin-layer chromatography (TLC) on 2.5 x 10-cm plates in two solvent systems. (a) Silica Gel H (Analtech, Inc., Newark, DE; not activated) developed in 48:40:10.5 (vol/vol) methanol/chloroform/water/concentrated NH4OH separated the inositides from the origin and resolved the three radioactive lipids (Fig. 1). Typical Rf values were phosphatidylinositol-4,5-bisphosphate, 0.38; phosphatidylinositol-4-phosphate, 0.46; lysophosphatidylcholine, 0.56; phosphatidic acid and heme, 0.60; sphingomyelin, phosphatidylserine, phosphatidylino- and lysophosphatidylethanolamine, 0.60-0.66; phosphatidylethanolamine, 0.72; phosphatidylethanolamine, 0.79; and cholesterol, 0.98. (b) Diacylglycerol was separated from other neutral lipids on Silica Gel G (Analtech; not activated) developed in 25:20:10:0.1 (vol/vol) benzene/diethyl ether/ethanol/acetic acid. Phospholipids remained at the origin; typical Rf values were unsaturated fatty acids, 0.48; cholesterol, 0.53; saturated fatty acids, 0.64; and diacylglycerol, 0.70. Lipid spots were detected with iodine vapor. Radiolabeled phospholipids were detected by autoradiography using Kodak X-Omat AR film (Eastman Kodak Co., Rochester, NY) with a Dupont Cronex intensifying screen (DuPont Instruments, Wilmington, DE) (Fig. 1e), and quantified by liquid scintillation counting of scraped spots.

Lipid phosphorus in phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate and total lipid phosphorus were determined by a small scale adaptation of the spectrophotometric method of Bartlett, corrected for silica background (27). No one-dimensional TLC system tested separated phosphatidylinositol and phosphatidic acid from the major phospholipids well enough to allow accurate phosphorus determinations. This separation was accomplished by two-dimensional TLC on 10 x 10 cm Silica Gel H (Analtech) plates. The solvent systems used were 65:25:5.5 (vol/vol) chloroform/methanol/concentrated NH4OH followed by 65:50:5 (vol/vol) chloroform/methanol/acetic acid to separate phosphatidylinositol (Fig. 1b), and 60:30:6 (vol/vol) chloroform/methanol/concentrated NH4OH followed by 60:10:6 (vol/vol) chloroform/methanol/acetic acid to separate phosphatidic acid (Fig. 1c). Both two-dimensional systems also afforded good separation of the major phospholipids.

RESULTS

Lipids were extracted from fresh human erythrocytes and the molar percentages of each phospholipid class were measured by phosphate assay. Table I summarizes the results of 6 one-dimensional and 24 two-dimensional TLC separations. The proportions of the major phospholipids agree well with previously reported values (28, 29) except for phosphatidylinositol-4,5-bisphosphate; whereas Allan and Michell found 0.4

Abreviations used in this paper. MI, morphological index; TLC, thin layer chromatography.
± 0.07%, we find 1.4 ± 0.3%. It is possible that their lower value reflects partial hydrolysis during their more extensive lysis and washing procedures (28).

As has been reported (28), red cells incubated with \(^{32}\)P-PO\(_4\) and nutrients incorporated detectable radiolabel into phosphatidylinositol-4-phosphate, phosphatidylinositol-4,5-bisphosphate, and phosphatidic acid, but not into other phospholipids (Fig. 1a). The radiolabel equilibrated with phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate in 15–25 h, approximately the time scale on which it equilibrates with ATP (15, 30). In contrast, phosphatidic acid equilibrated more slowly, with \(^{32}\)P-phosphatidic acid increasing steadily through 50 h of incubation even as ATP levels fell to a few percent of normal. This indicates that phosphatidic acid turnover is slow and that the diacylglycerol kinase remains active even at very low ATP levels.

The ratio of \(^{32}\)P-phosphatidylinositol-4,5-bisphosphate to \(^{32}\)P-phosphatidylinositol-4-phosphate fell somewhat during the prelabeling, indicating that the 5-phosphate equilibrates more rapidly than the 4-phosphate, in agreement with a previous report (31). The amounts of radiolabel incorporated into phosphatidylinositol-4,5-bisphosphate and phosphatidylinositol-4-phosphate at equilibrium indicate a molar ratio of 1.8 ± 0.3 (assuming two exchangeable phosphates per phosphatidylinositol-4,5-bisphosphate molecule and one per phosphatidylinositol-4-phosphate), in good agreement with the ratio obtained by phosphate assay (1.5 ± 0.4).

**Slow Metabolic Crenation**

Erythrocytes labeled with \(^{32}\)P were allowed to deplete their ATP slowly. After a lag of ~15 h, the cells began to crenate, leveling off at a morphological index of +3 after 45 h of incubation (Figs. 2 and 3). This shape change coincided with a 70% decrease in phosphatidylinositol-4,5-bisphosphate (Fig. 3b). Phosphatidylinositol-4-phosphate levels did not change significantly.

In separate experiments, unlabeled erythrocytes were allowed to deplete their ATP and their levels of phosphatidylinositol and phosphatidic acid were measured. After a lag, crenation proceeded at the same rate and to the same extent as for radiolabeled cells. The level of phosphatidylinositol nearly doubled, with its increase mirroring the previously noted drop in phosphatidylinositol-4,5-bisphosphate (Fig. 3b). Phosphatidic acid levels dropped by ~20%, accompanied by a qualitative increase in diacylglycerol. No changes in the levels of sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, lysophosphatidylcholine, or lysophosphatidylethanolamine were detected.

**Recovery of the Discoid Shape**

At two points during slow crenation, cells were pelleted, washed, and resuspended in supplemented NaCl/\(^{32}\)P. After
FIGURE 3  Slow metabolic crenation. (a) After cells were washed free of sugar, ATP levels fell (○) and the cells crenated (□, △). (b) As the cells changed shape, phosphatidylinositol-4,5-bisphosphate levels decreased (○) and phosphatidylinositol levels increased (△). Phosphatidic acid levels fell by a smaller amount (□) and phosphatidylinositol-4-phosphate fell only slightly (○). These data were taken from three experiments: one to assay ATP (○), shape (□), and radiolabeled phosphatidylinositol-4-phosphate (□) and phosphatidylinositol-4,5-bisphosphate (○); one to assay shape and phosphatidylinositol (△); and one to assay shape and phosphatidic acid (□). The zero time-point was taken to be the beginning of ATP depletion in the first experiment. In the other two experiments, the shape changes occurred at similar rates and to similar extents, but with somewhat different lag times. To facilitate comparison, the time scales for the latter experiments were shifted so that the shape changes from all three corresponded. In Figs. 3–5, the data shown for the radiolabeled phospholipids were calculated by converting \(^{32}P\) counts to moles of lipid (determined by phosphate assay), and then to percentage of total cell phospholipid. Error bars in this and all subsequent figures represent 1 SE.

Rapid Metabolic Crenation

Radiolabeled cells treated with inosine and iodoacetamide depleted their ATP in \(\sim 1\) h. As shown in Fig. 5, a decrease in phosphatidylinositol-4,5-bisphosphate once again coincided with crenation. Both the shape change (\(ΔMI = +2\)) and the decrease in phosphatidylinositol-4,5-bisphosphate (40%) were

FIGURE 2  (Top) Scanning electron micrographs of fresh discocytes (left) and echinocytes formed by 30 h of metabolic depletion (right). \(× 3000\). (Bottom) Morphology grading scale, based on the nomenclature of Bessis (22).

33 h of depletion, this nutrient supplementation allowed the cells to regenerate \(\sim 20\%\) of their ATP (Fig. 4a). Subsequently, the echinocytes (\(MI = +1.7\)) converted to discocytes and a few stage I stomatocytes (Fig. 4a). This shape recovery was accompanied by an increase in phosphatidylinositol-4,5-bisphosphate levels from \(\sim 85\%\) to 125% of normal, and a decrease in phosphatidylinositol-4-phosphate (Fig. 4b). There was no detectable change in phosphatidic acid levels.

A second nutrient supplementation was begun after 46 h of metabolic depletion. [ATP] rose more slowly and leveled off at 6% of normal (Fig. 4c), which was sufficient to allow the cells to revert from \(MI = +2.9\) to \(MI = +0.4\). The shape recovery began \(\sim 30\) min after the nutrients were added and leveled off after 3–4 h. After a similar time lag, phosphatidylinositol-4,5-bisphosphate levels rose to \(\sim 80\%\) of normal at 2 h (Fig. 4d). This increase was accompanied by an increase in phosphatidic acid to normal levels, and a decrease in phosphatidylinositol-4-phosphate levels.
DISCUSSION

Metabolism of Lipids during Metabolic Crenation and Shape Recovery

Fig. 6 summarizes the known metabolic pathways of phosphatidic acid and the inositides in human erythrocytes. At least two pairs of kinases and phosphatases interconvert phosphatidylinositol, phosphatidylinositol-4-phosphate, and phosphatidylinositol-4,5-bisphosphate (26, 31), and another pair interconverts diacylglycerol and phosphatidic acid (32, 33). In addition, both phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate can be hydrolyzed to diacylglycerol by a Ca$^{2+}$-dependent phospholipase C (28).

The absolute amount of phosphatidylinositol-4,5-bisphosphate degraded during slow metabolic depletion corresponds closely to the amount of phosphatidylinositol formed (Fig. 3), suggesting that phosphatidylinositol-4,5-bisphosphate is degraded by phosphomonesterases. This conclusion is supported by the previous demonstration that metabolic depletion engenders smaller increases in intracellular Ca$^{2+}$ (<1 μM) than the 100 μM required to activate the phospholipase C (34). Phosphatidylinositol-4-phosphate levels do not change, indicating that either the phosphatidylinositol-4,5-bisphosphate phosphatase and phosphatidylinositol-4-phosphate phosphatase operate at similar rates, or that phosphatidylinositol-4,5-bisphosphate is degraded to phosphatidylinositol by a route that does not involve phosphatidylinositol-4-phosphate (for example, conversion of phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-5-phosphate, followed by its rapid degradation to phosphatidylinositol). Slow crenation was also accompanied by a small decrease in phosphatidic acid, implying conversion to diacylglycerol via phosphatic acid phosphatae. Rapid ATP depletion effected more rapid and less extensive lipid degradation (Fig. 5), as well as a more rapid and less extensive shape change.

The lipid metabolism accompanying crenation appeared to be more or less reversed during shape recovery. Phosphatidic acid was restored to normal levels, and phosphatidylinositol-4,5-bisphosphate was restored to 125% (Fig. 4b) and 80% (Fig. 4d) of normal. The increase in phosphatidylinositol-4,5-bisphosphate levels was accompanied by a decrease in phosphatidylinositol-4-phosphate that was somewhat smaller in smaller than those observed in slow metabolic crenation, and both leveled off at ~8 h. Phosphatidic acid levels fell slightly, and phosphatidylinositol-4-phosphate levels appeared to remain constant.

![Figure 4](link) Shape recovery in crenated cells. (a) Erythrocytes were metabolically depleted for 33 h, then resuspended in supplemented NaCl/P; ATP levels rose (●) and the cells recovered their normal shape (○). (b) Lipid levels during the shape recovery showing an increase in phosphatidylinositol-4,5-bisphosphate (○), a decrease in phosphatidylinositol-4-phosphate (●), and no significant change in phosphatidic acid (□). (c) Erythrocytes were metabolically depleted for 46 h, then resuspended in supplemented NaCl/P; ATP level rose slightly (●) and the cells partially recovered their normal shape (○). During this shape recovery (d) phosphatidylinositol-4,5-bisphosphate (○) and phosphatidic acid (●) levels rose, while phosphatidylinositol-4-phosphate levels fell (□).

![Figure 5](link) Iodoacetamide-induced metabolic crenation. (a) ATP levels fell rapidly (●), and the cells crenated a few hours later (○). (b) Lipid levels changed more rapidly and less extensively than during slow metabolic crenation. Phosphatidylinositol-4,5-bisphosphate levels fell (○); phosphatidic acid fell slightly (□); and phosphatidylinositol-4-phosphate remained constant (●).

![Figure 6](link) Pathways of inositol metabolism. Three pairs of kinases and phosphomonesterases are thought to interconvert the inositides (PI, PI-P, PI-PP), diacylglycerol (DG), and phosphatidic acid (PA). High levels of intracellular calcium activate a Ca$^{2+}$-dependent phospholipase C, which degrades phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate to diacylglycerol. During metabolic crenation lipid metabolism is shifted in favor of diacylglycerol and phosphatidylinositol. The phospholipase C does not appear to be activated. During shape recovery in echinocytes, phosphatidylinositol-4,5-bisphosphate and phosphatidic acid are resynthesized.
absolute terms, implying that some phosphorylation of phosphatidylinositol was also taking place.

During both slow and rapid metabolic depletion, lipid changes paralleled the cell shape changes. During repletion, the shape recovery lagged perhaps 30–60 min behind lipid phosphorylation, still a close temporal association. The correlation between crenation and inositol breakdown also holds for the extremely rapid crenation induced by calcium loading. Allan and Thomas (20) reported that erythrocytes incubated with 1 mM Ca\(^{2+}\) and 5 \(\mu\)M A23187 (at hematocrit 20 in 130 mM NaCl/20 mM 3-[N-morpholino]propane sulfonic acid, pH 7.1) lose 70% of their phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate with a half-time of 4 min. Under the same conditions, we observe that cells crenate with a similar half-time of 2 min.

These results demonstrate that phosphatidylinositol-4,5-bisphosphate (and perhaps phosphatidic acid) is degraded or synthesized concomitantly with five shape changes whose characteristic times vary over three orders of magnitude (Table II). It is improbable that these associations are fortuitous, suggesting a causative link between lipid dephosphorylation and crenation. Further, inositol breakdown appears to mediate both metabolic crenation and Ca\(^{2+}\) crenation, but by different pathways: phosphonesterases in metabolic crenation and the Ca\(^{2+}\)-dependent phospholipase C in Ca\(^{2+}\) crenation.

### Lipid Changes and Bilayer Imbalance

Phosphatidylinositol-4,5-bisphosphate is thought to reside exclusively in the inner monolayer of the red cell membrane. It is highly polar and should flip-flop only very slowly. During crenation, phosphatidylinositol-4,5-bisphosphate is converted to phosphatidylinositol, which has a smaller, less highly charged headgroup. Thus, breakdown of phosphatidylinositol-4,5-bisphosphate would be expected to shrink the inner monolayer, even assuming that phosphatidylinositol, like phosphatidylinositol-4,5-bisphosphate, does not flip-flop. We can estimate roughly the amount of inner monolayer contraction this change should produce.

The concentration of phospholipids in packed red cells is 3.6 mM (35). Assuming a red cell volume of 88 fl, this concentration corresponds to 1.9 \(\times\) 10\(^8\) phospholipid molecules per cell, of which 1.4% or 2.7 \(\times\) 10\(^8\) molecules per cell are phosphatidylinositol-4,5-bisphosphate. About 70% of these, 1.3 \(\times\) 10\(^8\) molecules per cell, are degraded to phosphatidylinositol during slow metabolic crenation.

The membrane surface area normally occupied by a molecule of phosphatidylinositol-4,5-bisphosphate has not been measured. Based on x-ray diffraction studies of phosphatidylinositol-4,5-bisphosphate and phosphatidic acid, pH 7.1, lose 70% of their phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate with a half-time of 4 min. Under the same conditions, we observe that cells crenate with a similar half-time of 2 min.

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### Relationship to Shape Changes in Ghosts

When human erythrocytes are lysed in Tris buffer and resuspended in isotonic saline containing 1 mM MgATP at 0°C, they first crenate, then slowly recover their discoid shape (10), and eventually become stomatocytic. Several proposals have been made to explain ghost shape changes, some invoking rearrangements of the cytoskeleton (10, 41).

Unless Ca\(^{2+}\) is rigorously excluded, erythrocyte lysis is accompanied by breakdown of inositides and phosphatidic acid (26). This observation suggests that Tris lysis produces ghosts that are deficient in inner monolayer phospholipids, which could explain why they crenate at normal ionic strength. The recovery of the normal discoid shape upon incubation with MgATP could arise from resynthesis of the degraded lipids. The work of Fairbanks et al. (13) suggests that synthesis of phosphatidic acid and not phosphatidylinositol.
sitol-4,5-bisphosphate might be the key event in this recovery. Agents that produce crenation-resistant ghosts (17) might inhibit the degradation of phosphatidic acid or inositides, or might accelerate their resynthesis.

Relationship to Shape Changes in Platelets

The physiological roles of inositol metabolism and crenation in erythrocytes are not yet evident. However, they could be related to apparently similar phenomena that occur during platelet activation (42). Both cells change from smooth disks to spiculate spheres in response to Ca" loading. Platelets change shape in response to other stimuli as well, including platelet activating factor, a hydrophilic lecithin derivative that would be expected to cause rapid crenation in erythrocytes. In some cases, platelet activation has been correlated with the degradation of phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate. Both platelets and erythrocytes undergo their shape changes when unfixed cells are placed between a glass microscope slide and coverslip (unpublished observation). Finally, erythrocytes and platelets are derived from a common stem cell, and have similar phospholipid compositions and asymmetries (43). These similarities suggest that a common mechanism might underlie erythrocyte and platelet shape changes. Preliminary experiments in this laboratory suggest that platelet shape is governed by bilayer balance, and that agents that change platelet shape do so by disrupting this balance, either by physical intercalation or by inducing inositol breakdown.

We thank Kong Joo Lee and Katherine Mitchell for assistance with the scanning electron microscopy.

This work was supported by grants from the National Institutes of Health (HL 23787) and the American Heart Association (Grant-in-Aid 800990).

Received for publication 27 October 1983, and in revised form 2 February 1984.

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It should be emphasized that although reference 13 argues against a role for inositol phosphorylation in the echinocyte to discocyte shape change of ghosts, it does not conflict with the present work on intact cells.