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# Erythrocytes Attached to a Wheat Germ Agglutinin Coated Surface Display an Altered Phospholipid Metabolism

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Erythrocytes were bound to a lectin-coated surface; the multivalent attachment to this surface resulted in a severe deformation of the cells and an alteration in the cellular phospholipid metabolism. Human erythrocytes were allowed to bind for 20 min at 20°C to polystyrene beads coated with wheat germ agglutinin (WGA beads). The bound erythrocytes were then lysed to produce stroma bound to WGA beads. Control stroma and stroma-WGA beads were incubated at 37°C with $\gamma$-$^{32}$P-ATP to examine the phospholipid labeling patterns. The control stroma incorporated $^{32}$P-label into phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate, in agreement with earlier studies. However, the stroma-WGA beads showed incorporation of $^{32}$P-label into phosphatidic acid in addition to that in the phosphoinositides. The quantity of $^{32}$P-phosphatidic acid produced during the 20-min assay was 3.23 ± 0.84 (n = 7) picomoles/$\mu$g stromal cholesterol; the amount synthesized, however, was dependent on the procedure used to prepare the stroma-WGA beads. If the erythrocytes were bound to the WGA beads at 0°C instead of 20°C, the quantity of $^{32}$P-phosphatidic acid produced during the subsequent 37°C assay with $\gamma$-$^{32}$P-ATP was decreased 4.2 fold; the phosphoinositide labeling pattern was unchanged. In addition, when the time for binding of intact erythrocytes to the WGA beads was varied from 1 to 20 minutes, there was a time-dependent increase in the amount of $^{32}$P-phosphatidic acid produced. This induction of phosphatidic acid synthesis could not be duplicated with fluid phase WGA. Therefore, the multivalent binding of intact erythrocytes to WGA beads causes an alteration in phospholipid metabolism.

**Key words: lectin, phosphatidic acid**

The mature human erythrocyte has a rather limited capacity for phospholipid metabolism [1]. There is an active remodeling of existing phospholipids in the erythrocyte membrane, as demonstrated by fatty-acid exchange [1]; however, no de novo synthesis of phospholipids occurs [2]. The most active pathway in the mature erythrocyte involving phospholipid headgroups is an apparent "futile cycle" of phosphorylation/dephosphorylation of phosphoinositides [3,4]. In addition, Hokin and Hokin demonstrated that phosphatidic acid (PA) present in erythrocyte membranes may be hydrolyzed to dia-

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cylglycerol [5], which is then rephosphorylated to phosphatidic acid to serve as an additional cycle of phospholipid turnover. A calcium-activated phospholipase C that hydrolyzes phosphoinositides [6] is also present in the erythrocyte. This enzyme is not active with normal intracellular calcium levels; in fact, activation of this phospholipase would result in the irreversible loss of phosphoinositides by the erythrocyte.

Recent work has documented that complex interactions occur between phospholipids and the skeletal complex in the erythrocyte. Some of these observations have been very direct, such as the involvement of phosphatidylinositol-4,5-bisphosphate ($PIP_2$) in the association between glycophorin and protein 4.1 [7] or the effect of $PIP_2$ concentration on integral membrane protein lateral diffusion [8]. However, many reports have indirectly supported the concept of interdependencies between the membrane lipids and the skeletal complex. The best example of this is the demonstration of multiple metabolic pools of the phosphoinositides [9–11]. Because the phosphoinositides are located exclusively in the inner leaflet of the erythrocyte membrane, the mechanism for maintaining distinct pools of these lipids is unclear. However, most models probably would involve the skeletal complex in helping to define these domains. The size of these separate pools —none appear to be less than 25% of the $PIP_2$ present [11]—suggests that direct protein-lipid binding is not responsible for the metabolic heterogeneity.

Additionally, several workers have shown that the extracellular binding of antibodies or lectins to erythrocytes results in transmembrane modifications of the skeletal complex [12–14] or of the proteins associated with the complex [15]. It was with this background that we made the observation that lectin binding to the erythrocyte surface results in an alteration in the cellular phospholipid metabolism. The characterization of this reaction is the focus of our report.

## MATERIALS AND METHODS

Wheat germ agglutinin (WGA) was obtained from both Sigma Chemical Co., (St. Louis, MO) and Behring Diagnostics (La Jolla, CA). Concanavalin A, maclura pomifera, and sophora japonica were from Sigma Chemical Co. (St. Louis, MO). Polystyrene beads (no. 4023, approximately 75 μ diameter) were from Polysciences, Inc. (Warrington, PA), and $\gamma$-$^{32}$P-ATP was obtained from New England Nuclear (Boston, MA). DAG kinase was from Lipidex, Inc. (Middleton, WI). All other chemicals were of reagent grade or better.

### Attachment of Stroma to Beads

One hundred milligrams of dry polystyrene beads were placed in 17 × 100-mm polypropylene tubes (Falcon 2059); 3 ml of 0.1 mg/ml wheat germ agglutinin in PBS/EDTA (0.5 mM EDTA, 150 mM NaCl, 10 mM Na phosphate, pH 7.4) were added. The beads and solution were mixed by gentle agitation and pipetting until the beads were fully wetted, as indicated by the beads sinking to the bottom of the solution. The mixture was then occasionally agitated over 60 min. The beads were washed with PBS/EDTA by gentle centrifugation (300 rpm for 5 sec). Four washes were performed to insure the removal of soluble WGA. Approximately 90 μg of WGA binds to the 100 mg of beads, as determined with binding of $^{125}$I-WGA. The WGA beads were left in 4 ml of PBS/EDTA. Other lectins tested were attached to the polystyrene beads with the same protocol.

Fresh human erythrocytes were freed of platelets and leukocytes by previously published procedures [16]. The cells were washed three times in PBS/EDTA and left at a 20% hematocrit. One milliliter of these washed cells was then added to the tube of well suspended, WGA-coated polystyrene beads. This suspension was agitated intermittently by pipetting up and down with a plastic transfer pipette for 20 min at room temperature. The beads were then washed free of unbound erythrocytes by gently centrifuging the beads in excess PBS/EDTA. After two washings, the beads were treated with cold 10 mM Tris, 0.5 mM EGTA, pH 7.4 (lysing buffer). The beads were washed repeatedly with cold lysing buffer until white. The 100 mg of beads now containing coated stroma were left in 500 μl final volume.

### $^{32}$P-ATP Incubations

Five hundred microliters of 2 mM γ-$^{32}$P-ATP (2.4 μCi/μmole), 10 mM $MgCl_2$, 1 mM EGTA, 20 mM β-mercaptoethanol, and 50 mM imidazole, pH 7.4 ($^{32}$P-ATP mix) were added to the 500-μl suspension of stroma-WGA beads. This mixture was shaken at 37°C for 20 min. The reaction was terminated by the addition of 3 ml chloroform/methanol/concentrated HCl (20:40:1), followed by 1 ml water and 1 ml chloroform. This extraction mixture was mixed vigorously and then centrifuged gently. A spinal needle attached to a calibrated glass syringe was used to remove 1.5 ml of the lower chloroform phase. This chloroform phase was blown dry under a stream of nitrogen, quantitatively spotted onto a silica gel thin-layer chromatography plate with concentrating zone (E.M. No. 11845, E. Merck) and chromatographed in $CHCl_3/CH_3OH/3.3\ N\ NH_4OH$, 10 mM EDTA (50:40:12.5) [17]. Radioactive bands were localized, scraped and, quantitated as described before [18].

The following flow chart serves as a summary of the experimental details listed earlier.

A. Erythrocytes attached to WGA-beads; 20 min at 20°: INCUBATION 1
B. Beads washed free of unbound cells;
bound cells lysed and stroma-WGA beads washed.
C. Incubate stroma-WGA beads with γ-$^{32}$P-ATP: INCUBATION 2
D. Extract lipids and analyze by thin-layer chromatography.

### Intact Erythrocyte Incubations

Washed erythrocytes were suspended at a 20% hematocrit in 5 mM glucose, 0.15 M sodium chloride, 10 mM HEPES, pH 7.4, containing 5 μCi/ml $^{32}$P-inorganic phosphate. This mixture was incubated at 37°C for 180 min, after which the cells were washed and lysed as previously described [17].

### Stroma Incubations With γ-$^{32}$P-ATP

Fresh stroma were prepared by diluting washed erythrocytes into cold lysing buffer and centrifuging at 23,000g for 10 min at 4°C. The stroma were washed until white and left at a protein concentration of 2–3 mg/ml lysing buffer. One hundred microliters of this stroma preparation were incubated with 100 μl $^{32}$P-ATP mix, as detailed earlier. Extraction of the radioactive lipids and chromatography were done as detailed earlier.

### Cholesterol Determinations

After thin-layer chromatography of the phosphoinositide samples, the cholesterol band near the solvent front was sprayed with 6-p-toluidino-2-naphthalenesulfonic acid [19], visualized under an ultraviolet lamp, and scraped from the plate [18]. The silica gel with the bound cholesterol was dried on a lyophylizer, placed in a small glass column, pulverized, and extracted with 6 ml of $CHCl_3$/acetone (2:1). The solvent of this extract was blown down and cholesterol determined [20].

### Soluble WGA Incubations

Washed erythrocytes, $3 \times 10^8$, in 1.0 ml of PBS/EDTA were incubated with WGA (0.1–100 μg) for 20 min at 20°C. Stroma were then prepared and incubated with $^{32}$P-ATP as detailed. Radioactive PIP and $PIP_2$ were produced but no $^{32}$P-PA.

### Erythrocyte Lysis and Resealing

The procedure of Dale et al. [21] was used to lyse erythrocytes. Briefly, erythrocytes at a 60% hematocrit were placed in a dialysis bag and the bag added to a bottle of 0° 10 mM Tris, 0.5 mM EGTA, pH 7.4. The bottle with dialysis sac was rotated in a vertical plane at 0° for 60 min. After lysis was completed, the contents of the dialysis bag were aliquoted, and further dilutions of 1:2, 1:3 and 1:6 were made at 0° with the same lysis buffer. These final dilutions were left at 0° for 25 min before sufficient volume of 4 M NaCl was added to restore isotonicity. Erythrocytes were resealed at 37° for 15 min [21] and then washed three times with PBS/EDTA. Resealed erythrocytes were added to WGA beads as detailed earlier. The hemoglobin content of the resealed cells was determined by absorbance at 410 nm.

### Measurement of Diacylglycerol

The procedure of Preiss et al. [22] was followed. Washed erythrocyte stroma (approximately 200 μg protein) were extracted with 4 ml of $CHCl_3$/MEOH (2:1); 1 ml of 100 mM NaCl was added; and 2 ml of the lower phase was removed for analysis. Octyl glucoside, 1.5 mg, was added to this 2 ml of lower phase before blowing off the $CHCl_3$. Two hundred microliters of reaction mix (100 mM NaCl, 2 mM β-mercapto-ethanol, 10 mM $MgCl_2$, 1 mM EGTA, 75 mM imidazole, pH 6.6) were added to the dried extract and warmed at 37° for 10 min. The solubilized extract was then cooled to room temperature; excess DAG kinase and 1.5 mM $\gamma$-$^{32}$P-ATP (2.4 μCi/μmole) were added, and the reaction was continued at 20° for 45 min. Extraction and thin-layer chromatography of the $^{32}$P-PA product was performed as detailed earlier for the analysis of phosphoinositide. The quantity of $^{32}$P-PA produced was calculated on the basis of the $^{32}$P-ATP specific activity.

## RESULTS

The initial observation that prompted these studies is shown in Fig. 1. Lane A demonstrates the $^{32}$P-phospholipid labeling pattern for intact erythrocytes incubated with $^{32}$P-inorganic phosphate, where three radioactive bands are clearly seen representing $PIP_2$, PIP, and PA. Lane B represents the phospholipid labeling pattern when control stroma are incubated with $\gamma$-$^{32}$P-ATP; under these circumstances, only the phosphoinositides are labeled, in agreement with earlier observations [23–25]. However, when

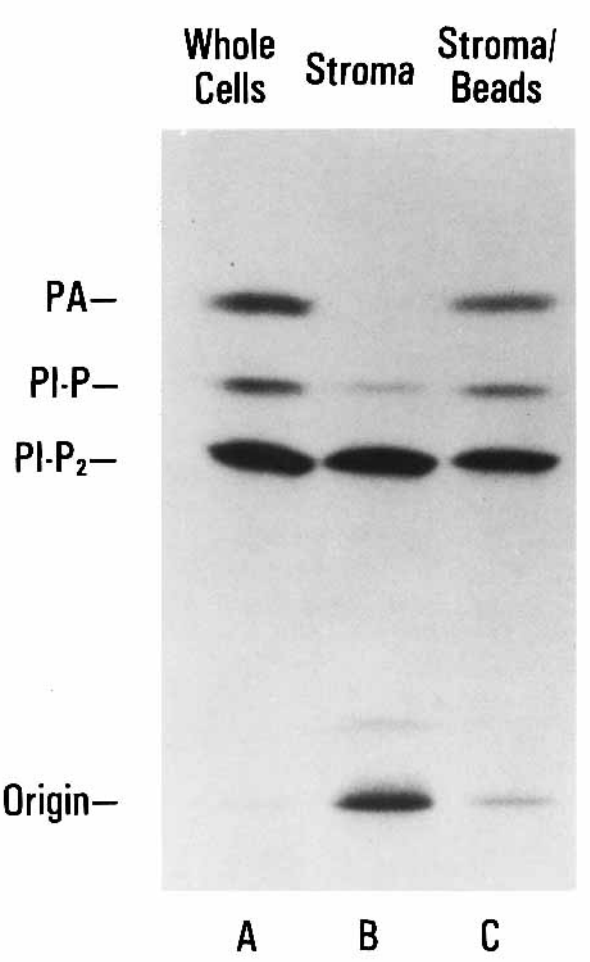


Fig. 1. Phospholipid labeling pattern of erythrocyte membranes. **Lane A:** Intact erythrocytes incubated with $^{32}$P-phosphate (see Methods); **lane B:** isolated stroma incubated with $^{32}$P-ATP; **lane C:** stroma attached to WGA beads and incubated with $^{32}$P-ATP. The radioactivity at the origin for lanes B and C represents $^{32}$P-ATP carried over during the lipid extraction.

erythrocytes are first immobilized onto a WGA-coated surface for 20 min at 20°C and then lysed and washed to produce white stroma bound to this surface, the phospholipid labeling pattern with $\gamma$-$^{32}$P-ATP for these stroma is altered, in that phosphatidic acid is also labeled (lane C).

Figure 2 shows erythrocytes bound to WGA beads. Panels A and B demonstrate that the bound erythrocytes are intact but severely deformed. Panel C depicts stroma-WGA beads produced after lysis of the bound cells.

The critical step that allows the stroma-WGA beads to synthesize $^{32}$P-phosphatidic acid is the binding and incubation of the intact erythrocytes on the WGA beads. This is shown in part by the effect of temperature on the system; a time course of $^{32}$P-incorporation into stroma-WGA beads prepared at two different temperatures is shown in Fig. 3. When the erythrocytes are bound to the WGA beads (incubation 1) at either

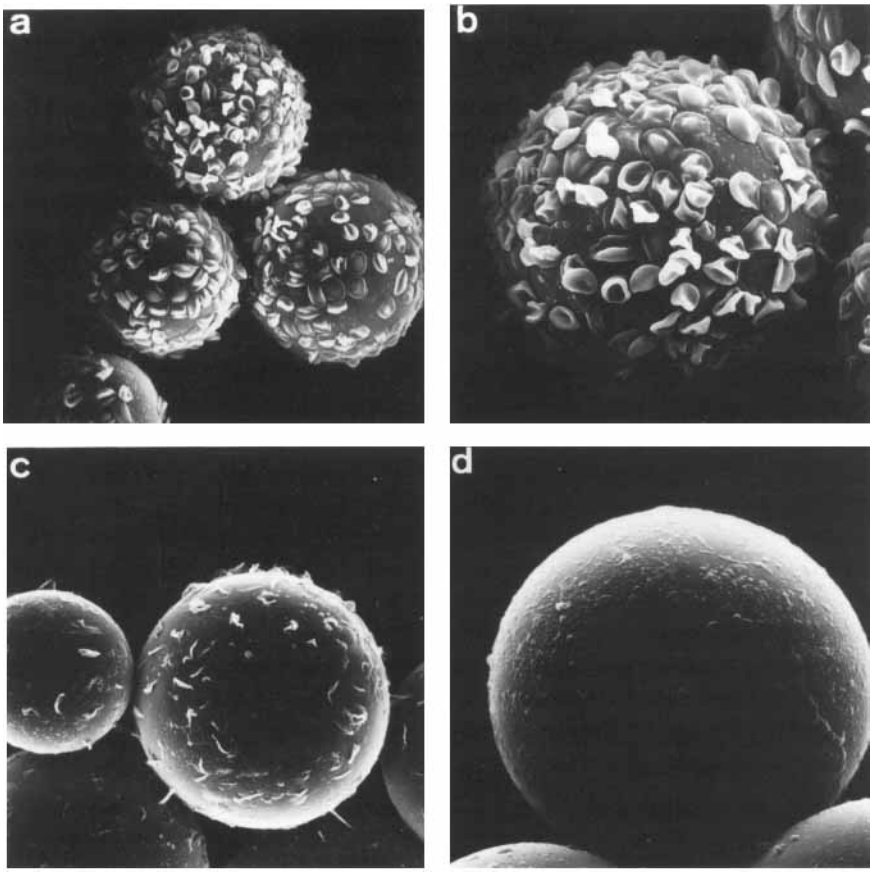


Fig. 2. Erythrocytes and stroma attached to wheat-germ-agglutinin–coated polystyrene beads. **Panel A:** Intact erythrocytes bound, X300; **panel B:** intact erythrocytes bound, X800; **panel C:** stroma on WGA beads, X600; **panel D:** uncoated beads, X800.

0°C or 20°C, the resulting stroma-WGA beads synthesize nearly identical amounts of PIP and $PIP_2$ in the subsequent 37°C assay with $\gamma$-$^{32}$P-ATP (incubation 2). However, the production of $^{32}$P-PA depends on the temperature used to prepare the stroma-WGA beads (incubation 1). The average synthesis of $^{32}$P-PA by the stroma-WGA beads produced at 20°C is 3.23 $\pm$ 0.84 (n = 7) picomoles/$\mu$g stromal cholesterol during the 20-min assay; the $^{32}$P-PA synthesis by the stroma-WGA beads produced at 0°C is 4.2-fold less. Because the stroma-WGA beads prepared at 0°C routinely bind 15–20% more membrane than do the 20°C beads, all the values shown in Fig. 3 are corrected for the amount of stromal cholesterol bound to the WGA beads.

Figure 4 shows the effect of time of incubation of the intact erythrocytes with the WGA beads at 20°C. Erythrocytes were incubated with WGA beads at 20°C for the time indicated and then washed and lysed to produce stroma-WGA beads. All preparations were then incubated with $\gamma^{32}$P-ATP for 20 min at 37°C. The data demonstrate that the production of $^{32}$P-PA is a function of the time that intact erythrocytes are incubated with the WGA beads. If erythrocytes are bound to WGA beads and then lysed, further

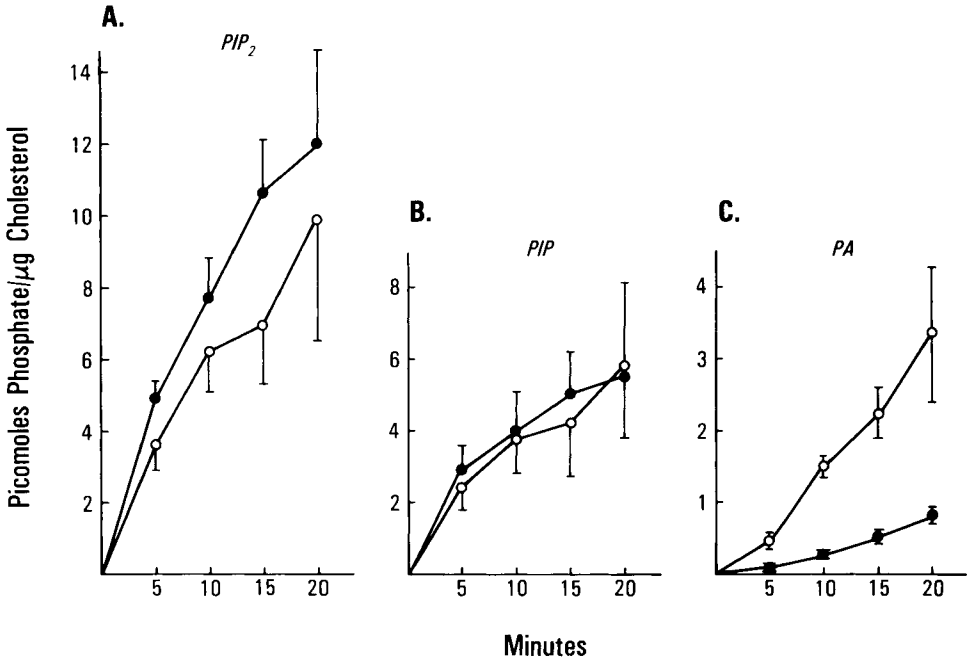


Fig. 3. Effect of erythrocyte-WGA bead-binding temperature on stroma-WGA beads. Erythrocytes were bound to WGA beads at 0°C (●) or 20°C (○) for 20 min. Stroma-WGA beads were then prepared and incubated with $^{32}$P-ATP at 37°C for the specified times. **Panel A:** $PIP_2$; **panel B:** PIP; **panel C:** PA.

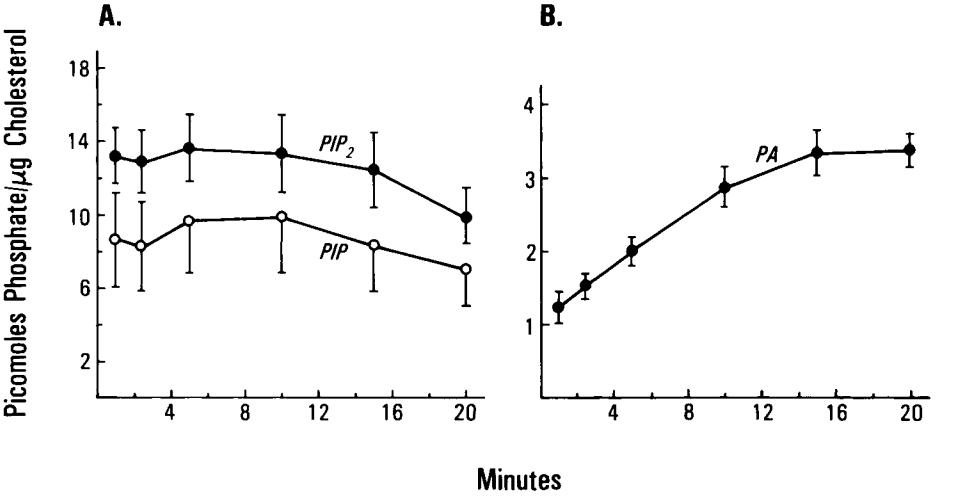


Fig. 4. Variation of erythrocyte-WGA-bead binding time. Intact erythrocytes were bound to WGA beads at 20°C for the specified times. Stroma-WGA beads were then prepared and incubated with $^{32}$P-ATP for 20 min at 37°C. **Panel A:** $PIP_2$ (●) and PIP (○); **panel B:** PA.

incubation of the washed stroma-WGA beads at 20°C does not result in increased production of $^{32}$P-PA in a final $^{32}$P-ATP assay incubation (data not shown). Therefore, the production of $^{32}$P-PA requires the binding and incubation of intact erythrocytes with WGA beads.

The effects described here cannot be duplicated by binding soluble WGA to erythrocytes. In three separate experiments, soluble WGA was added to erythrocytes, varying the WGA concentration over a thousandfold range, and no production of $^{32}$P-PA was seen, in contrast to the experiments described earlier (see Methods section). In addition, the presence of EDTA or EGTA during the erythrocyte attachment to and subsequent incubation with the WGA beads does not affect the generation of $^{32}$P-PA. This fact is critical because if the erythrocytes lysed while attached to the beads and if no calcium chelator were present, the calcium-dependent phospholipase C would be activated [6], resulting in the production of diacylglycerol and the subsequent synthesis of $^{32}$P-PA. However, the inclusion of up to 15 mM EGTA during the whole erythrocyte incubation with the WGA beads had no impact on the subsequent labeling.

Other lectins were tested for the ability to induce $^{32}$P-PA synthesis, as seen here with WGA beads. Figure 5 shows the results with three additional lectins; maclura pomifera, specific for glycophorin A; concanavalin A, specific for band 3; and sophora japonica, specific for type-A erythrocytes [26]. Whereas all three alternative lectins bind

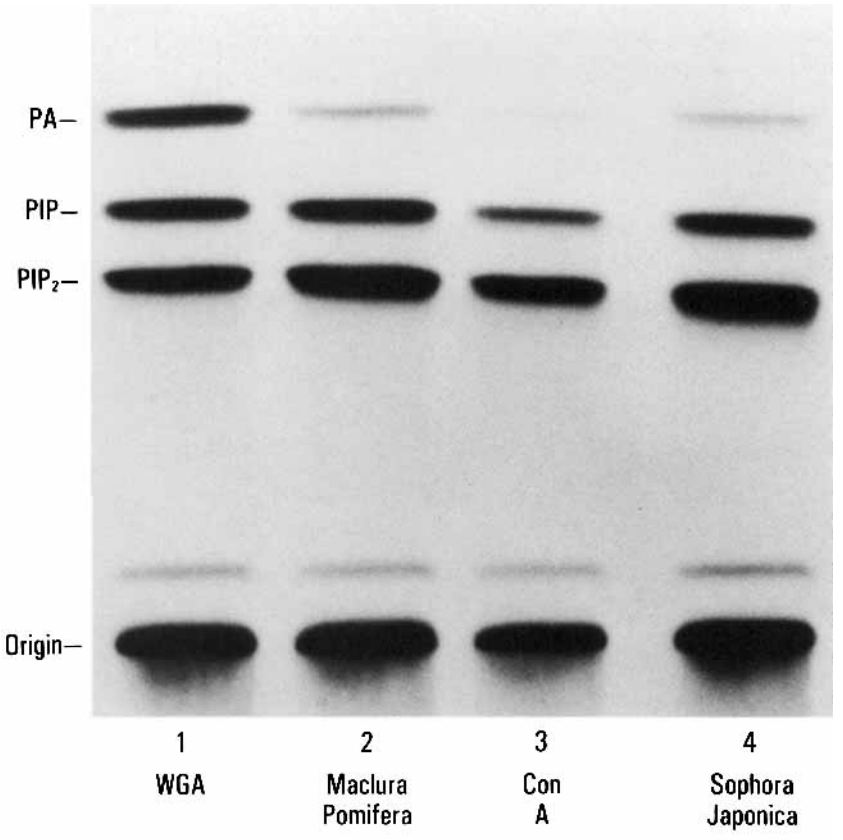


Fig. 5. Alternative lectins for the generation of $^{32}$P-PA. Intact erythrocytes were bound to lectin-coated beads as detailed in the text; in a subsequent $^{32}$P-ATP assay the ability of the stroma beads to synthesize $^{32}$P-PA was examined. **Lane 1** utilized the standard WGA beads, as shown in Fig. 1. **Lane 2** had maclura pomifera bound to the beads, **lane 3** used concanavalin A, and **lane 4** had sophora japonica on the beads. Only WGA allowed significant synthesis of $^{32}$P-PA.

erythrocytes, as shown by light microscopy and the synthesis here of $^{32}$P-PIP and $^{32}$P-$PIP_2$, none of them induces a significant synthesis of $^{32}$P-PA. Lanes 2 and 4, the maclura pomifera and sophora japonica, do induce some $^{32}$P-PA synthesis, but it is less than 15% of the amount induced by WGA, lane 1.

The nature of the reaction that results in the production of $^{32}$P-PA is partially revealed in Fig. 6. Erythrocytes were lysed by dialysis against a low-ionic-strength buffer at 0°; aliquots of the lysed cells were then diluted at 0° with increasing volumes of this same buffer before finally being resealed by raising the ionic strength and the temperature; see Methods. Each of these resealed erythrocyte preparations was then bound to WGA beads for 20 min at 20°, as detailed above, and the resulting stroma-WGA beads were then assayed in the presence of $^{32}$P-ATP. The data presented in Fig. 6 indicate that dilution of the erythrocyte's cytoplasmic contents, as measured by hemoglobin content, resulted in a decreased synthesis of $^{32}$P-PA. Extended dialysis (2 hr) during the initial lysis period of this experiment did not result in a diminution of the cell's ability to produce $^{32}$P-PA, suggesting that the critical component of this reaction is not a small molecule but a macromolecule.

The above data suggest that erythrocytes may produce diacylglycerol (DAG) during the incubation with WGA beads and that this DAG is subsequently converted to $^{32}$P-PA by DAG kinases and $^{32}$P-ATP. It was necessary, therefore, to measure DAG levels in the stroma-WGA bead preparations. DAG was measured by the method of Preiss et al. [22]. However, it was determined that control erythrocyte stroma have quite high levels of DAG, 4.46 ± 0.76 nmoles/mg stromal protein (mean ± SD; n = 8) or 73.8 picomoles/μg stromal cholesterol. These levels are approximately 20 times higher than the putative production seen in the presence of WGA beads (Fig. 3) and are of the same magnitude as earlier reports [27]. It is not clear why the DAG present in these control

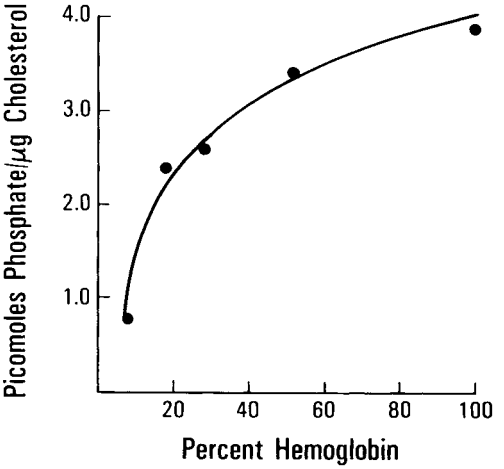


Fig. 6. Effect of dilution lysis of erythrocytes on WGA-bead reaction. Erythrocytes were lysed by a standard dialysis technique, diluted to varying degrees, and then resealed in an isotonic solution. The resulting resealed cells had decreased quantities of cytoplasmic components, as measured by hemoglobin retention. Each resealed erythrocyte preparation was bound to WGA beads for 20 min at 20° and subsequently assayed for the synthesis of $^{32}$P-PA. The data are expressed as picomoles of $^{32}$P-PA produced per microgram of cholesterol and are plotted vs. the percent of hemoglobin remaining in the lysed/resealed cells, compared with the starting erythrocytes.

membranes is not available to react with DAG kinase and $^{32}$P-ATP to produce $^{32}$P-PA; however, this conclusion is clearly supported by previous reports [23–25]. The ability of these control stroma to phosphorylate newly generated DAG is amply demonstrated by the inclusion of calcium to activate the endogenous phospholipase C activity [6] (data not shown).

## DISCUSSION

The data presented here demonstrate that the binding of intact erythrocytes to a lectin-coated surface results in an alteration in the cellular phospholipid metabolism. This alteration is demonstrated by the ability of these stroma-WGA beads to synthesize $^{32}$P-PA in the presence of $\gamma$-$^{32}$P-ATP. Neither control stroma nor soluble stroma with bound WGA will synthesize $^{32}$P-PA. Three alternative lectins were found to bind erythrocytes to the polystyrene beads, but these only weakly induced the formation of $^{32}$P-PA. There is no obvious pattern of selective membrane-protein binding by these lectins. WGA is known to bind glycophorin and band 3, maclura pomifera binds glycophorin, and con A binds to band 3. The one characteristic of WGA that separates it from the other lectins is the larger number of possible receptors on the surface of the erythrocyte, with the resulting opportunity for more interactions per unit of surface area when the erythrocyte binds to the WGA bead.

The nature of the reaction during incubation 1 is partially elucidated by the data in Fig. 6. In this experiment, dilution of the cytoplasmic contents of the resealed erythrocytes resulted in a progressive decrease in the ability of the stroma-WGA beads to synthesize $^{32}$P-PA in incubation 2. This observation together with the time and temperature dependence of incubation 1 would suggest that a soluble enzyme is present in the erythrocyte and either that the enzyme itself is activated by binding the cell to the WGA beads or that perhaps the enzyme's substrate becomes more available during this binding (incubation 1). Although the reaction catalyzed by this putative enzyme is not clear, one of its products may be DAG, which is subsequently measured as $^{32}$P-PA after the second incubation. Alternatively, the stroma-WGA beads may simply be more efficient at phosphorylating endogenous DAG. Attempts to measure increased DAG generated during incubation 1 were thwarted by the high endogenous level of DAG present in the erythrocyte membrane.

The most striking features of the immobilized erythrocytes shown in Fig. 2 are their abnormal shapes; they are clearly deformed by the multivalent attachment to the WGA surface. One possible explanation for the data presented here would be that a normal erythrocyte maintains domains or compartments of some lipids and that these compartments prevent the enzymatic action of active phospholipases or kinases, whereas in the present experiments, the multivalent attachment of the erythrocyte to the WGA beads may be disrupting lipid domains and allowing phospholipases or kinases to have access to normally unavailable substrates. The end result of these alterations would be the generation of $^{32}$P-PA.

## ACKNOWLEDGMENTS

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