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Research Article

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Alteration of Membrane Phospholipid Bilayer Organization in Human Erythrocytes during Drug-induced Endocytosis

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A B S T R A C T   Our plan was to evaluate the potentially important role of phospholipids in erythrocyte shape alterations by determining if their orientation was altered during endocytosis. Stomatocytosis and endocytosis were induced in normal intact human erythrocytes by incubation with three agents: primaquine, vinblastine, and chlorpromazine, each of which has its own requirements and time course for producing endocytosis. The organization of the phospholipid bilayer was assessed by measuring the extent of degradation of phophatidylcholine (PC), phophatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (SM) produced by exposure of erythrocytes to a nonpenetrating protease-free phospholipase A_2 alone or in combination with a purified sphingomyelinase as well. The induction of stomatocytosis did not change this orientation. However, correlating with the onset of endocytosis but not its extent, there was an increase in PE degradation, which could be detected regularly only by use of phospholipase A_2 alone. Use of the combination of phospholipase A_2 and sphingomyelinase showed that the extent and course of endocytosis was paralleled by an apparent movement of PC and SM from the outer to the inner half of the lipid bilayer. Since no further PE was hydrolyzed and because no PS was ever degraded, this inward movement of PC and SM did not represent the establishment of complete symmetry in the membrane. By adjusting the experimental design it was possible to implicate the endocytic process, and not insertion of drug in the membrane, as the cause of the alterations in phospholipid organization seen. Our findings indicate that the phospholipid orientation is very closely involved in the endocytosis process and that specific states of phospholipid asymmetry may be related to identifiable membrane events.

INTRODUCTION

The vectorial aspects of the human erythrocyte (RBC)[1] as well as its shape is thought to be determined, in part, by the asymmetric distribution of proteins across the membrane (1-3). Studies with both small molecular probes like trinitrobenzenesulfonate (4, 5) and nonpenetrating enzymes (6-8) provide generally consistent data showing that the phospholipids in the bilayer are also asymmetrically arranged, with sphingomyelin (SM) and phosphatidylcholine (PC) being located primarily in the outer leaflet while the aminophospholipids, phosphatidylethanolamine (PE) and phosphatidylserine (PS), being located at the inner leaflet. The negatively charged PS, in particular, seems to be totally restricted to the inner half of the bilayer.

We decided to evaluate the potential role of phospholipids in RBC shape alterations by determining if the orientation of these phospholipids was altered during endocytosis. Endocytosis in intact adult RBC can be produced by incubation with amphipathic cations like primaquine, chlorpromazine, and vinblastine (9). Each agent quickly produces stomatocytosis (10),

[1] *Abbreviations used in this paper:* PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; RBC, erythrocyte(s); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SM, sphingomyelin; vit B_{12}, vitamin B_{12}.

which is followed in a variable period of time by endocytosis, a process that involves membrane invagination, constriction, and fusion (11). These three drugs have very different requirements for endocytosis in terms of their need for ATP and the anionic and cationic contents of the incubation media (11, 12). The entry and distribution of these agents into RBC has also been studied (13). By manipulating this system and making serial measurements of endocytosis while determining in parallel the accessibility of the constituents of the phospholipid bilayer to nonpenetrating enzymatic probes, we thought we might be able to determine if changes in phospholipid organization accompanied either stomatocytosis and/or endocytosis, and in particular, examine the effects of endocytosis on phospholipid organization within the bilayer.

In evaluating phospholipid bilayer organization in RBC undergoing stomatocytosis and endocytosis, the cells were initially exposed to a purified protease-free, nonpenetrating bee venom phospholipase A_2, which degrades only the glycerophospholipids located in the outer leaflet of the lipid bilayer. Usually 60% of the PC and 0–10% of the PE are hydrolyzed following such treatment. More complete information about phospholipid orientation is provided by treating RBC first with phospholipase A_2 and subsequently with sphingomyelinase. In addition to hydrolyzing SM this combined enzymatic treatment hydrolyzes all of the PC and PE located in the outer membrane leaflet. However, this attack does not degrade any PS, which appears to be confined entirely to the inner leaflet. Based upon this combined enzymatic approach it is generally believed that 75% of PC, 20% of PE, and 80% of SM are located in the outer leaflet of the bilayer. The results of our study indicate that significant alterations in phospholipid organization accompany endocytosis, but not stomatocytosis, and that particular domains of PE and PC may be involved in the endocytic process.

METHODS

Materials. The materials for measuring the extent of RBC endocytosis (14), measuring [^{3}H]chlorpromazine entry into RBC (13), performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15), and for measuring phospholipid asymmetry were obtained from previously described sources (5, 8).

Procedures. All experiments were performed on freshly drawn heparinized venous blood provided by normal volunteer donors under protocols established and approved by the Stanford Committee on Human Experimentation and by the Oakland Children's Hospital Medical Center Committee. The plasma and buffy coat were removed and the plasma was retained for subsequent use in the endocytosis experiments. The RBC were then washed, once at 4°C in 0.145 M NaCl and three times with Hanks' balanced salt solution (HBSS), also at 4°C.

Measurement of stomatocytosis and endocytosis was performed as previously described (14). Briefly, 1 vol of washed, packed RBC in HBSS (hematocrit ~75%) was mixed with 1 vol of HBSS containing the endocytosis-inducing agent and 1 vol of autologous plasma containing [^{57}Co]vitamin (vit) B_{12}. Incubation was carried out at 37°C, for varying periods of time. The trapping of the [^{57}Co]vit B_{12}-vit B_{12}-binding protein complex with endocytic vacuoles provided a quantitative measurement of endocytosis (14). Confirmation of endocytosis and evaluation of shape change was routinely done by phase-microscopic examination of coded samples. When experiments were performed in isotonic sodium lactate or isotonic sodium glutamate, all washes were done with either isotonic sodium lactate or isotonic glutamate buffered to pH 7.4. In such experiments, the autologous plasma had been previously extensively dialyzed against the same isotonic lactate or glutamate supplemented with 5 mM Ca^{2+} and 2 mg glucose/ml. The appropriate lactate or glutamate solution then replaced the HBSS regularly used. The reaction mixtures were shaken for 5 min at 37°C before the endocytosis-inducing drugs were added. Upon addition of drug, samples were immediately withdrawn and centrifuged for 2 min in the Brinkmann 3200 centrifuge (Brinkmann Instruments, Inc., Westbury, NY) for initial evaluation of RBC shape, endocytosis, and phospholipid bilayer organization. Approximately 5 min were required for isolation of this initial sample, which is called the "0 time sample." Measurement of [^{3}H]chlorpromazine entry into RBC was performed as previously described (13).

Assessment of RBC membrane phospholipid organization was performed by using phospholipase A_2 from bee venom and sphingomyelinase C from *Staphylococcus aureus* (8). In brief, the 0.25-ml aliquot of drug treated or control RBC was suspended in 5 ml of 5 mM phosphate-buffered saline (PBS) (290 mosM) containing 5 mM KCl, 1 mM $MgSO_4$, and 0.5 mM $CaCl_2$, pH 7.4. For the single enzyme treatment experiments, 30 IU of phospholipase A_2 from bee venom or 10 IU of sphingomyelinase from *S. aureus* was added to appropriate samples and incubated at 37°C for 1 or 2 h. For the double-enzyme treatment experiments, appropriate erythrocyte samples were first incubated with 30 IU of phospholipase A_2 from bee venom for 1 h, followed by incubation with 10 IU of sphingomyelinase C for an additional hour. The degradation of phospholipid by phospholipase A_2 or the combination of phospholipase A_2 and sphingomyelinase was terminated by washing the RBC three times with PBS containing 5 mM EDTA. The extent of hemolysis was determined at the end of each incubation before the EDTA wash by comparing the hemoglobin content in the supernate of each sample to that of a 100% hemolyzed control. Hemolysis was found to be <7% in all experiments reported in this paper. To determine the extent of phospholipid degradation, phospholipase-treated and control RBC samples were washed with PBS and subjected to lipid extraction by the method of Rose and Oklander (16). Lipid extracts from each sample were evaporated to dryness under nitrogen and redissolved in a small volume (100–200 μl) of 2:1 chloroform/methanol mixture. Individual phospholipids were separated by the two-dimensional thin-layer chromatographic technique described by Roelofsen and Zwaal (17). The individual lipid components were examined by staining with iodine vapor. All spots were scraped from the plate and transferred to test tubes, and the quantity of phospholipid was determined by measuring the amount of phosphorus in each spot, using the method of Bottcher et al. (18). The percentage of phospholipid hydrolyzed after treatment of RBC with phospholipase A_2 was determined by measuring the ratio of remaining diacylglyc-

erophospholipid to the corresponding lyso derivative. The specificity of phospholipase A_2 toward pure lipid substrates in an isolated system was not altered in the presence of either primaquine, chlorpromazine, or vinblastine in the reaction mixture. For the determination of SM degradation by sphingomyelinase, the absolute and relative quantity of SM recovered from the sample was compared with the absolute and relative quantity of SM recovered from the nontreated control sample. Since neither of these enzymes penetrate the lipid bilayer (17), hydrolysis of phospholipids can be interpreted to indicate location in the outer leaflet of the bilayer.

RESULTS

All three drugs produced prompt and extensive stomatocytosis at the 0 time sample as identified by phase microscopy. Primaquine endocytosis measured by the ^{57}Co method and by phase microscopy was dose and time dependent (Fig. 1). However, there was a 30-min lag period between the essentially immediate microscopic appearance of extensive stomatocytosis and the onset of endocytosis as detected either by the radioisotopic method or by morphologic examination. PE hydrolysis detected by the bee venom phospholipase A_2 increased in conjunction with or in anticipation of endocytosis and occurred long after the stomatocytic shape change had taken place. However, the extent of endocytosis produced did not correlate with extent of PE hydrolysis by phospholipase A_2. Using vinblastine the results were similar in that the increase in PE hydrolysis produced by exposure to the phospholipase

A_2 occurred with the onset of endocytosis (Fig. 2) and not the appearance of stomatocytosis. Chlorpromazine always produces very rapid endocytosis, which is essentially complete by the time the initial samples can be isolated and processed (12, 19). Increasing concentrations of chlorpromazine (Fig. 3) produced increasing amount of endocytosis. The increased accessibility of PE to hydrolysis by phospholipase A_2 alone matched the time course of endocytosis but not its extent.

The hydrolysis of PC by phospholipase A_2 seemingly did not change during either the stomatocytosis or the endocytosis produced (Table I). Thus, an increase in PE degradation by phospholipase A_2 was not matched by a reciprocal decrease in PC degradation, a circumstance that could have occurred if symmetry were to be developed in the bilayer.

To obtain a more complete picture of phospholipid organization during drug-induced endocytosis these experiments were repeated using sequential degradation of erythrocytes first with phospholipase A_2 and then sphingomyelinase, a procedure that could hydrolyze all of the PC, PE, and SM located in the outer lipid leaflet. Both PC and SM became progressively inaccessible to hydrolysis as primaquine endocytosis proceeded (Table I). This primaquine-induced inaccessibility of PC and SM to phospholipases was not related to incomplete phospholipase degradation, because prolonged incubation (2 h) of primaquine-treated RBC with phospholipases did not result in more PC nor SM degradation than that shown in Table I (data not shown). PS was never hydrolyzed by the combined
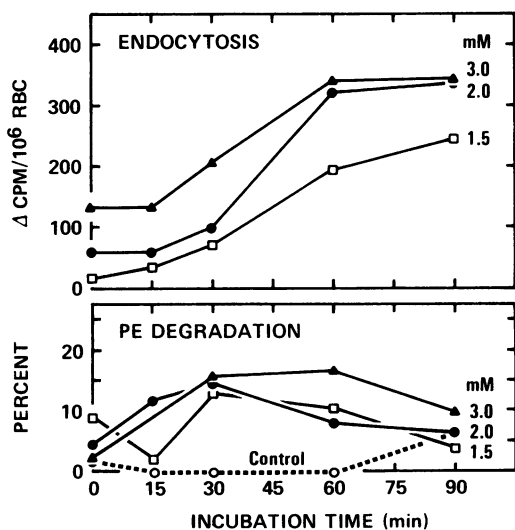

FIGURE 1 Primaquine endocytosis at three concentrations is shown on the *upper* graph as Δcounts per minute of [^{57}Co]vit B_{12} trapped in 10^6 RBC. Accessibility of PE to phospholipase A_2 hydrolysis is shown in parallel in the *lower* graph as percentage of PE degraded. The dotted line indicates values for control RBC incubated without primaquine.
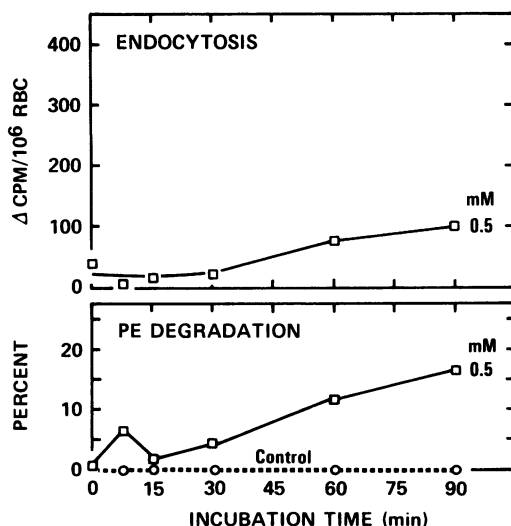

FIGURE 2 Vinblastine endocytosis at 0.5 mM is shown on the *upper* panel. The percentage of PE degradation from the same samples is shown in the *lower* panel as in Fig. 1. Control RBC were incubated in parallel without addition of drug.
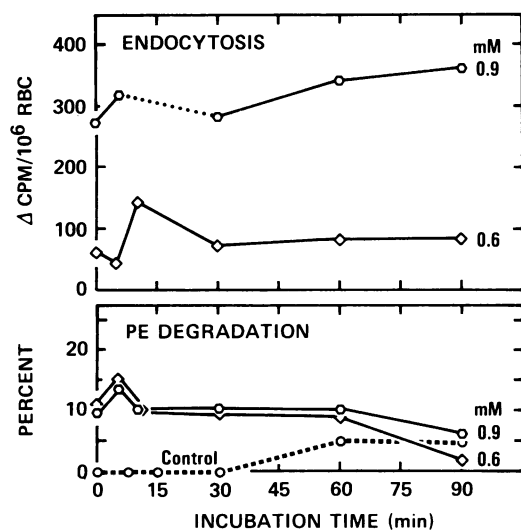
FIGURE 3 Chlorpromazine endocytosis is shown for two concentrations in the *upper* panel. The *lower* panel records the percentage of PE degraded at the indicated time points along with the values for RBC incubated without drug as in Figs. 1 and 2.

enzymatic treatment, and this observation indicates that PS remained confined to the inner lipid leaflet. The increase in PE hydrolysis detected by the phospholipase A_2 alone (Table I) again was correlated with the onset of endocytosis and not with the extent of endocytosis or its progression over time. A consistent further increase in PE hydrolysis following endocytosis was not seen using both enzymes.

For reasons still unknown to us, the treatment of erythrocytes with phospholipase A_2 followed by sphingomyelinase did not produce complete hydrolysis of outer leaflet sphingomyelin (80–85%) under our present experimental condition. To ensure that the decreased

SM degradation following drug treatment is not related to incomplete degradation, but to endocytosis, we determined SM accessibility in erythrocytes treated with primaquine, chlorpromazine, or velban using sphingomyelinase C alone. The results (Table II) of this experiment clearly indicate that accompanying drug-induced endocytosis was a decrease in SM accessibility to sphingomyelinase in the outer lipid leaflet. The extent of decrease is similar to that observed in the combined enzyme treatment (Tables I and III).

These changes in phospholipid organization could have been caused by the insertion of amphiphatic drug into the membrane, or alternatively they could have been the result or the cause of the endocytic process. To resolve these alternatives we relied on our prior observations showing that the extent of endocytosis produced by a given concentration of an agent could be enhanced or suppressed by altering the anionic composition of the suspending medium (14).

RBC were incubated with 2 mM primaquine suspended in either HBSS, sodium lactate, or sodium glutamate. As anticipated, lactate enhanced endocytosis while glutamate completely blocked it (Fig. 4) (14). Combined phospholipase A_2 and sphingomyelinase attack (Table III) revealed decreasing accessibility of SM and PC to hydrolysis and this decrease was correlating with both the course and extent of endocytosis in HBSS-suspended RBC. There was a sharper decrease in hydrolysis of both SM and PC in erythrocytes suspended in lactate. This greater inaccessibility of SM and PC in lactate was correlated with the enhanced endocytosis seen in isotonic lactate (Fig. 4). Despite the fact that there was more than twice as much endocytosis in lactate as in HBSS-suspended RBC at 30 min of incubation (Fig. 4), the increase in PE hydrolysis detected by phospholipase A_2 alone was equal in both, again suggesting that the increased accessibility of PE to phospholipase A_2 alone correlated with the onset of en-

TABLE I

Endocytosis with 2 mM Primaquine: Accessibility of Phospholipids

	Time of incubation		
	0	30 min	60 min
		%	
SM°	64	63	55
PC°	75	70	65
PS°	0	0	0
PE°	23	20	28
PC‡	64	63	61
PE‡	7	18	16

° Treatment with phospholipase A_2 and sphingomyelinase.
‡ Treatment with phospholipase A_2 alone.

TABLE II

Accessibility of SM to Sphingomyelinase C (S. aureus) in Erythrocytes Treated with Endocytic Drugs

RBS sample	SM degradation
	%
Control	85
Primaquine treated (2 mM, 60 min)°	72
Chlorpromazine treated (1.0 mM, 5 min)°	70
Vinblastine treated (0.5 mM, 90 min)°	70

° The values in parentheses indicate the drug concentration and the time of incubation of RBC with that particular drug, respectively.

Accessibility of Phospholipids to Phospholipase A_2 and
Sphingomyelinase C in Erythrocytes Treated with 2 mM
Primaquine and with or without Lactate or Glutamate

		Phospholipid degradation		
	n	0 time	60 min	
		%		
HBSS				
SM*	4	67±6.8	51±9.0	P < 0.01
PC*	4	76±1.3	62±3.8	P < 0.01
PE‡	4	6.4±3.3	17±3.6	P < 0.01
PE*	4	21±1.4	21±4.7	NS
Lactate§				
SM*		56, 68	40, 50	
PC*		74, 76	50, 56	
PE‡		5.0, 8.6	14, 18	
PE*		19, 21	17, 21	
Glutamate				
SM*	4	65±9.7	61±11.7	NS
PC*	4	77±1.7	74±2.0	NS
PE‡	4	7.3±3.8	6.4±4.1	NS
PE*	4	20±2.0	22±2.0	NS

Data represent mean±1 SD.
* Phospholipase A_2 then sphingomyelinase C.
‡ Phospholipase A_2 alone.
§ Values of two separate experiments are indicated.

FIGURE 4 The extent of endocytosis produced by 2 mM primaquine is indicated for three different suspending media.

docytosis and not its extent. Glutamate blocked the endocytosis usually produced by 2 mM primaquine and in parallel seemingly blocked alterations in phospholipid bilayer organization.

This experiment tended to implicate endocytosis rather than drug insertion as the cause of the alterations in phospholipid organization. However, because it is technically difficult to make measurements of membrane-bound primaquine (12), it remained possible that suspension of RBC in lactate or glutamate could have altered the entry and membrane binding of primaquine. Therefore, these experiments were re-peated using chlorpromazine, because the availability of [³H]chlorpromazine has made it possible to accurately measure its entry and distribution within RBC (13). Glutamate inhibited chlorpromazine endocytosis (Fig. 5). [³H]Chlorpromazine entered RBC equally from HBSS or glutamate media and the fraction of [³H]chlorpromazine recovered from the cytosol and membrane fraction were the same in both (Table IV). The reduction in endocytosis produced by glutamate was paralleled by a diminution in all of the alterations in phospholipid bilayer organization previously noted. The decrease in hydrolysis of PC and SM by phospholipase A_2 and sphingomyelinase was not detectable
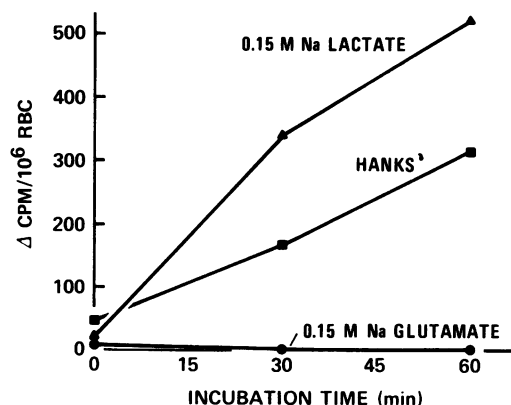
in glutamate (Fig. 5). Similarly the increase in PE hydrolysis by phospholipase A_2 was diminished by glutamate (Fig. 5). Therefore, the alterations in phospholipid bilayer organization observed appear to be related to the endocytic process, and not to the intercalation of drug in the membrane.

DISCUSSION

Incubation of adult human RBC with three classes of amphipathic cations produces almost immediate stomatocytosis. After varying periods of time, endocytic vacuoles appear, usually but not invariably, at the site of the advancing and deepening stoma (11). Our aim was to evaluate the role of the organization of the phospholipid bilayer in the stomatocytic shape change and in the general membrane properties of invagination, constriction, and fusion, which are involved in endocytosis. The fact that several classes of drug produce these changes over varying time frames provided an opportunity to study the role of phospholipid bilayer organization in these membrane-related events under circumstances where the effects of the insertion of drug could be distinguished from the subsequent steps involved in the endocytic process.

In exploring the organization of the phospholipid bilayer, we used nonpenetrating enzymatic probes in a manner similar to the approach used by us for the study of RBC membrane phospholipid asymmetry during the sickling process (8). Exploration of the orientation and organization of the phospholipid bilayer has been technically difficult, in part because the probes used could alter the bilayer in the process of exploring and reporting its organization. Nevertheless, small molecular probe analysis like that provided by trinitrobenzenesulfonate and the use of nonpenetrating enzymes both yield consistent data indicating that

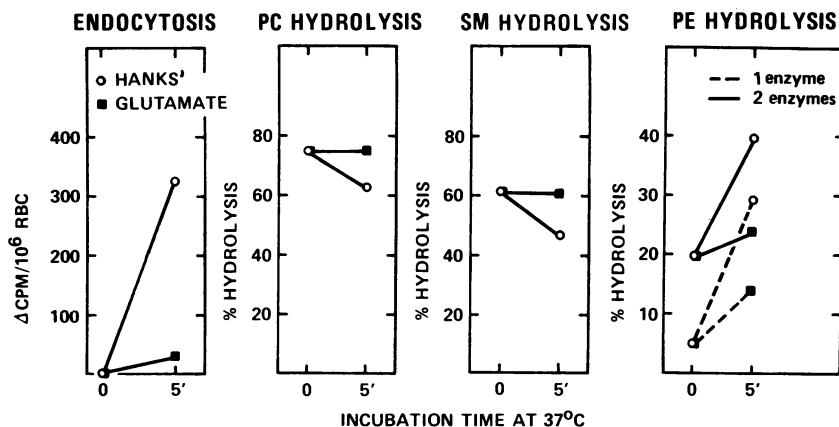## ENDOCYTOSIS   PC HYDROLYSIS   SM HYDROLYSIS   PE HYDROLYSIS

FIGURE 5 Four vertical panels are shown indicating the impact of chlorpromazine endocytosis on the phospholipid bilayer organization of the erythrocyte membrane. In the first vertical panel on the extreme left the extent of endocytosis over a 5-min period is indicated for 0.9 mM chlorpromazine, where the erythrocytes were suspended in either isotonic HBSS (O) or isotonic sodium glutamate (■). The second vertical panel from the left indicates the extent of PC hydrolyzed in these circumstances using combined enzymatic hydrolysis. The third vertical panel indicates the extent of SM hydrolysis using combined enzymatic hydrolysis. The fourth vertical panel (extreme right) indicates the extent of PE hydrolysis seen under these circumstances. The dotted lines indicated the results obtained when the erythrocytes were exposed only to the phospholipase A_2, whereas the solid lines indicate the results obtained when the erythrocytes were treated sequentially with phospholipase A_2 and then sphingomyelinase.

PC and SM are primarily localized (~75%) in the outer half of the bilayer, while all of the PS and 80% of the PE are in the inner or cytosol facing half of the bilayer (4–7).

No alterations in phospholipid bilayer asymmetry were detected in association with stomatocytosis. This is consistent with our reported studies on phospholipid organization performed on erythrocytes from a patient with hereditary stomatocytosis (20). All changes in phospholipid organization are therefore presumably related to the several steps in endocytosis. Each of the three agents used gave essentially identical results. The agents used vary in their chemical structure and in

their requirements for producing endocytosis. For example, chlorpromazine produces endocytosis essentially instantaneously and does not require energy in the form of RBC ATP (12, 19), while primaquine produces endocytosis over a 30–60-min period (Fig. I) and has an absolute requirement for erythrocyte ATP (12, 14). Therefore, the drug used was less important than the endocytic process in producing the alterations of phospholipid bilayer asymmetry noted.

The changes in phospholipid bilayer organization appeared to occur in the following sequence. In anticipation of, or coincident with the onset of endocytosis, accessibility of PE to hydrolysis by bee venom

TABLE IV
Uptake of [^{3}H]Chlorpromazine in RBC Suspended in HBSS or Glutamate

Concentration of [^{3}H]chlorpromazine	Time of incubation	Donor I		Donor II	
		HBSS	Glutamate	HBSS	Glutamate
	min				
0.71 mM	5	330* (18)‡	321 (8)	360 (14)	332 (38)
	10	273 (5)	357 (6)	312 (16)	319 (19)
0.15 mM	5	74 (12)	67 (7)	77 (12)	70 (7)
	10	65 (8)	68 (8)	70 (8)	64 (8)

* Values are expressed as nanomoles [^{3}H]chlorpromazine taken up per milliliter packed RBC.
‡ Numbers in parentheses refer to the fraction of [^{3}H]chlorpromazine in the washed RBC recovered in a membrane pellet produced by freezing and thawing.

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phospholipase A_2 increased. The detection of this increase in PE accessibility was regularly demonstrated when the phospholipase A_2 probe was used alone under circumstances where usually 0–10% of PE is accessible. The enhancement of PE accessibility was not regularly seen when the combined enzymatic treatment with phospholipase A_2 and sphingomyelinase was used (Table I). Under these latter circumstances 20–25% of PE is accessible to enzymatic degradation in control erythrocytes and this amount is not increased further as a consequence of endocytosis. This observation suggested that a subclass of PE in the outer half of the bilayer became exposed coincident with endocytosis and that use of combined enzymatic attack might have obliterated the selectivity that allowed the detection of the PE subclass involved. The idea that there may be subpopulations of PE within the membrane is supported by a recent report (21) showing that a small subpopulation of PE in the outer leaflet had a much higher turnover rate than the remainder of PE. The extent of PE accessibility to the phospholipase A_2 was not paralleled by the extent of endocytosis produced. Therefore, the increase in PE accessibility appears to reflect the trigger or switch that accompanies the onset of endocytosis. Perhaps the PE that becomes accessible to the phospholipase A_2 may be all of the PE in that subclass, which is made available in an all or none manner in association with the onset of endocytosis.

In contrast to the behavior of PE, both externally oriented PC and SM became similarly less accessible with the onset of endocytosis, and the decrease in accessibility paralleled the extent of endocytosis, becoming progressively inaccessible as endocytosis continued. This reduction in PC and SM accessibility was not caused by drug intercalation alone, because in the face of normal uptake and distribution of [^{3}H]chlorpromazine, but with endocytosis blocked by glutamate these alterations did not occur (Fig. 5 and Table IV).

The decreased accessibility of PC and SM to degradation could have been the result of bulk inaccessibility of membrane phospholipids trapped in endocytic vacuoles, which were now in the cytosol, shielded from the hydrolytic enzymes by the intact erythrocyte plasma membrane. However, "trapping" cannot account for all the observations we made. Firstly, using phospholipase A_2 alone in our system did not reveal any decrease in PC degradation (Table I). Secondly, although the combined enzyme treatment showed decreased PC and SM degradation, PE degradation remained fairly constant (Table III). Lastly, if trapped in the endocytic vesicles, the phospholipases could still be active in degrading phospholipids. Taken together, these observations suggest that the decreased PC and SM degradation by combined enzymatic treatment (Table III) should not be explained as a simple consequence of bulk inaccessibility of membrane phospholipids trapped in endocytic vacuoles.

Although the progressive inaccessibility of both SM and PC during endocytosis could reflect PC and SM movement toward the inner half of the lipid bilayer, such movement of PC was only detected by combined enzymatic degradation (Tables I–III) and not when phospholipase A_2 was used alone (Table I). Therefore, it is likely that endocytosis involves a subclass of PC that interacts with SM, and there is new evidence that there are domains of PC in human erythrocyte membranes (22). However, because this progressive loss in accessibility of SM and PC that accompanied endocytosis was not matched by a continuing increase in accessibility of PE, nor by any accessibility of PS, these changes do not represent complete loss of bilayer asymmetry.

Furthermore, the continued inability to detect any PS accessibility argues that the interrelationship between spectrin and PS is not perturbed during endocytosis, in contrast to the increase in PS accessibility seen with sickling (8) or oxidant damage (23, 24), where there probably is alteration of this relationship (23, 24). We searched for evidence of spectrin cross-linking using nonreducing 6% SDS–PAGE gels (23, 24) and found none (data not shown).

During the invasion of rhesus RBC by *Plasmodium knowlesi* it was noted that there was a decrease in accessibility of PC while PE became more accessible to the phospholipase A_2 used. PS remained completely inaccessible to either chemical or enzymatic probes during the parasitic invasion (25). These results are quite similar to ours. It is known that the process of plasmodial invasion of RBC requires the fixation of the malarial parasite to a specific membrane receptor site, following which there is a convulsive endocytosis of the entire merozoite (26). On the basis of our observations we would conclude that the changes reported in parasitized RBC reflect the endocytic process by which the malarial parasite entered the erythrocyte.

In summary, there are striking alterations in the membrane phospholipid bilayer organization that occur during endocytosis but not stomatocytosis. The changes seen are not simply produced by the intercalation of drug into the membrane. There is an increase in accessibility to bee venom phospholipase A_2 of a subclass of PE that precedes endocytosis and does not correlate with its extent or progression. Then, correlating with the extent and progression of endocytosis, there appears to be an inward movement of SM along with a subclass of PC, which is probably closely associated with SM. This inward movement of SM and PC does not indicate complete loss of bilayer asymmetry, since there is no outward movement of PS.

The importance of these observations relates to sev-

eral aspects of RBC membrane biology. Our results support the concept, based on chemical data, (21, 22) that erythrocyte membrane PC and PE do not represent single pools of phospholipid, but each is probably made up of subclasses of these compounds residing in specific domains. Furthermore, our studies imply that these putative subclasses of PE and PC have functional importance. Recent evidence suggests that a critical step involved in membrane fusion (an important step in endocytosis) is the hexagonal phase transition within the bilayer structure (27). Subclasses of phospholipids may be involved in this hexagonal form, and such changes may affect accessibility of membrane phospholipids to phospholipase degradation as a consequence of involvement in the endocytic process. Our data also shows that membrane phospholipids can no longer be considered as existing only in two organizations, asymmetrical and symmetrical. In fact, there may be several levels of asymmetry reflecting a variety of membrane events, and we believe we have identified one such state and its association with endocytosis.

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