Registry No. Ca, 7440-70-2; IP_3, 88269-39-0.

REFERENCES

Berridge, M. J. (1987) *Annu. Rev. Biochem. 56*, 159-193.
Berridge, M. J., Cobbold, P. H., & Cuthbertson, K. S. (1988) *Philos. Trans. R. Soc. London, B 320*, 325-343.
Carafoli, E. (1987) *Annu. Rev. Biochem. 56*, 395-433.
Cobbold, P., Daly, M., Dixon, J., & Woods, N. (1989) *Biochem. Soc. Trans. 17*, 9-10.
Colquhoun, D., & Sakmann, B. (1985) *J. Physiol. 369*, 501-557.
Guillemette, G., Balla, T., Baukal, A. J., & Catt, K. J. (1988) *J. Biol. Chem. 263*, 4541-4548.
Jacob, R., Merritt, J. E., Hallam, T. J., & Rink, T. J. (1988) *Nature 355*, 40-45.
Karpen, J. W., Zimmerman, A. L., Stryer, L., & Baylor, D. A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1287-1291.
Meyer, T., & Stryer, L. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5051-5055.
Meyer, T., Holowka, D., & Stryer, L. (1988) *Science 240*, 653-656.
Putney, J. W., Jr. (1987) *Am. J. Physiol. 252*, G149-G157.
Sage, S. O., & Rink, T. J. (1987) *J. Biol. Chem. 262*, 16364-16369.
Spät, A., Bradford, P. G., McKinney, J. S., Rubin, R. P., & Putney, J. W., Jr. (1986) *Nature 319*, 514-516.
Supattapone, S., Worley, J. M., Baraban, S. H., & Snyder, J. (1988) *J. Biol. Chem. 263*, 1530-1536.
Taurog, J. D., Fewtrell, C., & Becker, E. L. (1979) *J. Immunol. 122*, 2150-2153.
Walker, J. W., Somlyo, A. V., Goldman, Y. E., Somlyo, A. P., & Trentham, D. R. (1987) *Nature 327*, 249-252.
Woods, N. M., Cuthbertson, K. S., & Cobbold, P. H. (1986) *Nature 319*, 600-602.

Aminophospholipid Translocation in Erythrocytes: Evidence for the Involvement of a Specific Transporter and an Endofacial Protein†

Jerome Connor and Alan J. Schroit*

Department of Cell Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Received July 5, 1989; Revised Manuscript Received August 11, 1989

ABSTRACT: The transport of exogenously supplied fluorescent analogues of aminophospholipids from the outer to inner leaflet in red blood cells (RBC) is dependent upon the oxidative status of membrane sulfhydryls. Oxidation of a sulfhydryl on a 32-kDa membrane protein by pyridyldithioethylamine (PDA) has been previously shown [Connor & Schroit (1988) *Biochemistry 27*, 848-851] to inhibit the transport of NBD-labeled phosphatidylserine (NBD-PS). In the present study, other sulfhydryl oxidants were examined to determine whether additional sites are involved in the transport process. Our results show that diamide inhibits the transport of NBD-PS via a mechanism that is independent of the 32-kDa site. This is shown by the inability of diamide to block labeling of the 32-kDa sulfhydryl with ^{125}I-labeled PDA and to protect against PDA-mediated inhibition of NBD-PS transport. Diamide-mediated inhibition, but not PDA-mediated inhibition, could be reversed by reduction with cysteamine or endogenous glutathione. Similarly, treatment of RBC with 5,5′-dithiobis(2-nitrobenzoic acid), which depletes endogenous glutathione and induces oxidation of endofacial proteins [Reglinski et al. (1988) *J. Biol. Chem. 263*, 12360-12366], inhibited NBD-PS transport in a manner analogous to diamide. Once established, the asymmetric distribution of NBD-PS could not be altered by oxidation of either site. These data indicate that a second site critical to the transport of aminophospholipids resides on the endofacial surface and suggest that the transport of aminophospholipids across the bilayer membrane of RBC depends on a coordinated and complementary process between a cytoskeletal component and the 32-kDa membrane polypeptide; both must be operative for transport to proceed.

Phospholipids are asymmetrically distributed in the human erythrocyte membrane (Rothman & Lenard, 1977; Op den Kamp, 1979). In these cells, the aminophospholipids phosphatidylserine (PS)[1] and phosphatidylethanolamine preferentially reside in the inner leaflet, whereas the choline phospholipids are localized in the outer leaflet (Verkleij et al., 1973; Gordesky et al., 1975). This asymmetric organization is maintained through complex and, at present, poorly understood interactions between specific membrane proteins. On the one hand, it has been shown that endofacial proteins such as spectrin (Haest, 1982; Cohen et al., 1986; Maksymiw et al., 1987) and possibly band 4.1 (Sato & Ohnishi, 1983; Rybicki et al., 1988; Cohen et al., 1988; Shiffer et al., 1988) can stabilize PS in the cell's inner leaflet, whereas an ATP-dependent translocase is responsible for the outside to inside

† This work was supported by Grants CA-47845 and DK-41714 from the National Institutes of Health and by Developmental Fund Grant 175416 from The University of Texas M. D. Anderson Cancer Center.
* To whom correspondence should be addressed.

[1] Abbreviations: ^{125}I-PDA, *N*-[3-(3-[^{125}I]iodo-4-hydroxyphenyl)-propionyl]pyridyldithioethylamine (Bolton-Hunter-labeled PDA); DOPC, dioleoylphosphatidylcholine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent); DTT, dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione; NBD-PS, 1-oleoyl-2-[[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylserine; PBS, phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 10 mM sodium/potassium phosphates, and 10 mM glucose, pH 7.4); NEM, *N*-ethylmaleimide; PC, phosphatidylcholine; PDA, pyridyldithioethylamine; PS, phosphatidylserine; RBC, red blood cell(s); SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

0006-2960/90/0429-37$02.50/0

movement of exogenously inserted PS analogues (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985). Thus, it appears that two mechanisms may be responsible for the maintenance of PS asymmetry in human RBC: a direct interaction of PS with the membrane skeleton (Haest & Deuticke, 1976; Haest et al., 1978; Dressler et al., 1984) and an ATP-dependent translocation of PS from the outer toward the inner membrane leaflet (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985; Tilley et al., 1986).

Although the actual mechanism(s) responsible for PS translocation and its maintenance in the cells' inner leaflet has (have) not been fully established, many of the requirements influencing the cells' ability to maintain lipid asymmetry have been determined. For example, diamide-induced oxidative cross-linking of spectrin (Haest et al., 1978) inhibits the translocation of exogenously inserted PS analogues (Connor & Schroit, 1987). In addition, the transbilayer movement of PS is temperature and ATP dependent and requires that membrane protein sulfhydryls be maintained in the reduced state (Connor & Schroit, 1988). In this context, recent work from this laboratory has demonstrated that the protein most likely to be responsible for the transbilayer movement of PS is a 32-kDa membrane polypeptide (Schroit et al., 1987; Connor & Schroit, 1988). While this polypeptide appears to participate in the transbilayer movement of PS, it is not known whether it functions independently or in concert with cytoskeletal proteins that bind PS (Williamson et al., 1987).

In this report, we examined the effects of two classes of inhibitors of PS translocation: PDA, which oxidizes a free sulfhydryl on the 32-kDa membrane polypeptide, and diamide or DTNB, both of which interact, directly or indirectly, with a site distinct from that of the 32-kDa polypeptide. In contrast to the PDA site, the diamide/DTNB site is susceptible to reduction by endogenous glutathione and exogenously supplied cysteamine, indicating that a second site critical to the transport process resides on the endofacial surface. These results suggest that the oxidative status of the cytoskeleton, in addition to that of the 32-kDa polypeptide, serves an essential role in the maintenance of PS asymmetry.

Experimental Procedures

Materials and Routine Procedures. NBD-PC and DOPC were purchased from Avanti Polar Lipids (Birmingham, AL). NBD-PS was prepared from NBD-PC by phospholipase D catalyzed base exchange in the presence of L-serine (Comfurius & Zwaal, 1977) and purified by thin-layer chromatography. Diamide, iodoacetamide, NEM, mersalyl acid, DTT, cysteamine, and GSH were purchased from Sigma Chemical Co. (St. Louis, MO). ^{125}I Bolton–Hunter reagent (specific activity ~2000 Ci/mmol) was purchased from New England Nuclear (Boston, MA). PDA and ^{125}I-PDA were synthesized as previously described (Connor & Schroit, 1988). Human RBC were obtained from healthy volunteers by venipuncture into heparinized saline. The cells were washed 3 times with large volumes of PBS buffer and labeled with ^{51}Cr. Steady-state fluorescence was measured at 535 nm (λ_{ex} 468 nm) with a Farrand MK II spectrophotofluorometer at room temperature using 10-nm slit widths. Acceptor vesicles were prepared by ultrasonication of DOPC (10 mg/mL) in PBS. There was <5% deviation between the results of individual experiments which were repeated from 5 to 10 times by different personnel.

Inhibition of NBD-Lipid Transport. RBC (2×10^8 cells/mL) were incubated with the indicated inhibitors as described in the table legends. After two washes with PBS, the RBC were diluted to 2×10^7 cells/mL, added to NBD-lipid [100 ng of lipid (10 μL of EtOH)$^{-1}$ (mL of RBC)$^{-1}$] in the presence or absence of DTT (5 mM), cysteamine (2.5 mM), or GSH (10 mM), and incubated at 37 °C for 45 min. The ability of the cells to transport NBD-PS from the outer to inner leaflet was determined by assessing the residual fluorescence (lipid transported to the inner leaflet) of RBC "back-exchanged" with 0.5 mg of DOPC acceptor vesicles for 45 min at 0 °C. Acceptor vesicles were removed by centrifugation, and the cells were solubilized in 1% Triton X-100. The fraction of NBD-lipid remaining in the cells after this procedure was determined by fluorescence using aliquots of cells not subjected to back-exchange as controls. All fluorescent measurements were normalized for variations in cell number (<10%) based on ^{51}Cr counts of the final detergent lysates. To determine whether any of the inhibitors oxidized the sulfhydryl critical to PS transport on the 32-kDa polypeptide, aliquots of inhibitor-treated RBC were incubated with ^{125}I-PDA for 30 min at 4 °C. After the RBC were washed, ghosts were prepared by using 5 mM phosphate buffer (Steck, 1974). The ability of ^{125}I-PDA to label the transporter was determined by autoradiography after separation of the ghost proteins by SDS–PAGE using a 12% separating gel and a 6% stacking gel with the discontinuous buffer system of Laemmli (1970). The gels were stained with Coomassie R-250, dried, and autoradiographed with Kodak XAR-5 X-ray film.

Treatment of RBC with Multiple Inhibitors. RBC (2×10^8 cells/mL) were incubated with PDA or diamide for 30 min as described above. The cells were then thoroughly washed and treated a second time with PDA, diamide, NEM, or iodoacetamide under the same conditions employed during the single-reagent incubations. The cells were then washed, and their ability to transport NBD-PS in the absence or presence of DTT (5 mM) or cysteamine (2.5 mM) was determined as described above.

Measurement of ATP and Reduced and Oxidized Glutathione in Control and Inhibitor-Treated Cells. At different times after the indicated treatments, RBC were washed with PBS and resuspended to 2×10^8 cells/mL. ATP content was determined in 100 μL of RBC (2×10^7 cells) using the Sigma bioluminescent (luciferase/luciferin) assay kit (FL-ASC) as described by the manufacturer. The intensity of the peak flash was detected by a photomultiplier with continuous recording. Standard curves prepared using known amounts of ATP were linear in the range of interest. GSH content of RBC was determined essentially as described by Beutler et al. (1963). Briefly, a pellet of 2×10^9 cells was suspended in 1.0 mL of ice-cold water; 0.25 mL of 10% trichloroacetic acid (TCA) was added, the samples were centrifuged at 15000*g* for 3 min, and 0.5 mL of the supernatant was transferred to new tubes that contained 1.0 mL of 0.3 M Na_2HPO_4; 0.5 mL of DTNB (1 mM in 1% potassium citrate) was added, and the absorbance was measured at 412 nm using GSH as a standard. The amount of GSSG was determined by a procedure slightly modified from that described by Habeeb (1972). Briefly, 0.35 mL of the TCA-precipitated supernatant was mixed with 0.65 mL of 0.3 M Na_2HPO_4 and 1.0 mL of 1.6% $NaBH_4$ in water. The samples were then incubated at 37 °C for 30 min. Excess borohydride was destroyed by adding 1.0 mL of 1 M KH_2PO_4/0.6 N HCl. After 10 min, 1.0 mL of 2 M Tris base and 1.0 mL of the DTNB reagent were added. Absorbance was determined at 412 nm using GSH as a standard. The fraction of GSSG was calculated from the difference in the amounts of GSH and combined GSH/GSSG determined by both procedures.

Depletion of GSH and DTNB-Induced Protein Cross-Linking. Ellman's reagent was used to induce an oxidative

Table I: Effects of Sulfhydryl-Reactive Reagents on the Transbilayer Movement of NBD-PS[a]

inhibitor[b]	translocation (%)		
	NBD-PC	NBD-PS	NBD-PS + DTT
control (no treatment)	11.5	90.8	93.6
NEM	14.0	16.6	20.1
iodoacetamide	13.9	11.3	15.0
diamide	10.6	17.1	93.1
PDA	17.1	17.8	94.6
DTNB	11.2	88.5	
mersalyl	13.2	83.8	
azide/deoxyglucose	10.3	20.8	

[a] RBC (2×10^8/mL) were incubated with the indicated inhibitors for 30 min. The cells were then washed and incubated with NBD-PC or NBD-PS [100 ng (10 μL of ETOH)$^{-1}$ (2×10^7 RBC/mL)$^{-1}$] for 45 min at 37 °C in the absence or presence of DTT (5 mM). The fraction of NBD-labeled lipid remaining in the cells was then determined as described under Experimental Procedures. The values are from a representative experiment repeated 5–10 times. [b] RBC were incubated with the inhibitors under the following conditions: NEM (2 mM) at 4 °C; iodoacetamide (10 mM) at 37 °C; diamide (5 mM) at 20 °C; PDA (2 mM) at 4 °C; DTNB (5 mM) at 4 °C; mersalyl (1 mM) at 4 °C. ATP was depleted by incubating the RBC in the presence of deoxyglucose (50 mM) and azide (5 mM) for 3 h at 37 °C in the absence of glucose.

stimulus on the exofacial membrane sulfhydryl groups of RBC by thiol–disulfide exchange as described by Reglinski et al. (1988). Briefly, RBC (2×10^8 cells/mL) were treated with DTNB (5 mM) in PBS without glucose at 37 °C. At various times, aliquots were removed, washed free of DTNB, and assessed for their GSH and ATP content and their ability to transport NBD-PS in the presence or absence of DTT, cysteamine, glucose, and extracellular GSH. DTNB (and diamide) induced high molecular weight complex formation was determined by SDS–PAGE using 5% and 3% running and stacking gels, respectively.

Outward Translocation of NBD-PS. To determine whether NBD-PS, which resides in the inner leaflet of RBC, could be translocated to the outer leaflet (flop), cells were incubated with NBD-PS for 1 h at 37 °C [2×10^7 cells (100 ng of NBD-PS)$^{-1}$ (10 μL of EtOH)$^{-1}$ (mL of RBC)$^{-1}$]. The RBC were washed to remove unincorporated lipid and then mixed with DOPC acceptor vesicles (0.5 mg/mL RBC) for 45 min at 0 °C to remove any residual NBD-PS occupying the outer leaflet. These cells, which contained NBD-PS only in their inner leaflets, were then assessed for the appearance of NBD-PS in their outer leaflets after a 1-h incubation at 37 °C in the presence or absence of the various inhibitors. This was accomplished by determining the fraction of NBD-PS remaining in the cells (lipid not transported from the inner to outer leaflet) after an additional back-exchange with DOPC acceptor vesicles as described above.

Results

Effect of Sulfhydryl Oxidizing Reagents on the Transbilayer Movement of NBD-PS. The ability of PS (Daleke & Huestis, 1985; Tilley et al., 1986) and its spin-labeled (Seigneuret & Devaux, 1984) or fluorescent analogues (Connor & Schroit, 1987, 1988) to translocate from the outer to inner leaflets in RBC has been shown to be ATP dependent and to require that protein sulfhydryls be in the reduced state. The results shown in Table I indicate that the sulfhydryl critical to the transbilayer movement of NBD-PS is not at the membrane surface. This can be seen by the inability of DTNB and mersalyl, which do not penetrate the bilayer membrane, to inhibit the transport of NBD-PS. This finding confirms our previous result which showed that the addition of reduced

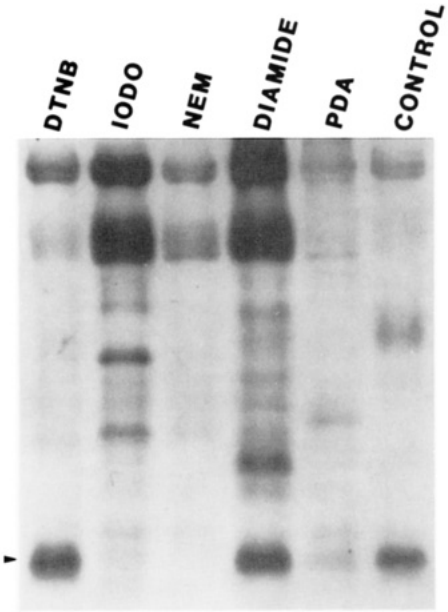

FIGURE 1: Labeling of the 32-kDa polypeptide by ^{125}I-PDA after RBC treatment with sulfhydryl oxidants. RBC were treated with the indicated inhibitors as described in Table I. The RBC were then washed and labeled with ^{125}I-PDA for 30 min at 0 °C. The cells were washed twice, ghosts were prepared with 5 mM phosphate (pH 8), and the membranes were solubilized in SDS without reduction. Approximately 2×10^8 RBC equiv was applied to each lane of a 12% gel. Arrow denotes 32-kDa polypeptide.

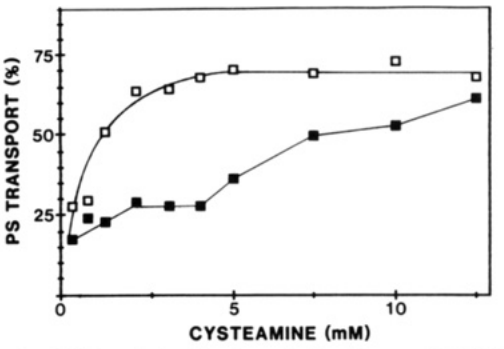

FIGURE 2: Differential reduction of diamide- and PDA-induced inhibition of NBD-PS transport by cysteamine. RBC were treated with diamide or PDA as described in Table I. Increasing concentrations of cysteamine were then added, and the ability of the cells to transport NBD-PS was determined after incubation at 37 °C for 45 min. (■) PDA-treated RBC; (□) diamide-treated RBC.

glutathione, in contrast to DTT, did not reverse the inhibition of NBD-PS transport by "reversible" sulfhydryl oxidants (Connor & Schroit, 1988).

To determine whether the oxidizing reagents shown in Table I react with the 32-kDa polypeptide (Connor & Schroit, 1988), RBC were treated with the various inhibitors, and the ability of the sulfhydryl on the 32-kDa polypeptide to be labeled with ^{125}I-PDA was determined by autoradiography. SDS–PAGE analysis of ghosts from these RBC revealed that the membrane-permeable reagents NEM and iodoacetamide completely blocked ^{125}I-PDA labeling of the transporter (Figure 1). A more complex picture emerged in the case of diamide. Although it effectively and reversibly inhibited PS transport (Table I), it did not inhibit ^{125}I-PDA labeling of the 32-kDa polypeptide. This result suggests that the inhibition of PS transport induced by diamide occurs at a site and by a mechanism that is independent of the 32-kDa sulfhydryl.

PDA and Diamide Inhibit the Transbilayer Movement of NBD-PS at Independent Sites. If indeed two distinct sites are

Table II: Transport of NBD-PS following Multiple Treatments with Inhibitors of PS Transport[a]

primary treatment	secondary treatment	tertiary treatment	NBD-PS transport (%) control	DTT	cysteamine
NT[b]			88.8	92.7	82.8
PDA			17.8	91.5	30.3
diamide			20.8	84.1	63.0
NEM			22.8	28.4	24.9
iodoacetamide			15.4	24.8	14.9
PDA	NT		18.3	91.4	24.2
PDA	PDA		14.1	91.2	21.7
PDA	diamide		15.2	82.2	24.4
PDA	diamide	NEM	21.7	66.3	
PDA	NEM		12.1	64.0	22.4
PDA	iodoacetamide		14.1	27.1	23.1
diamide	NT		30.6	84.4	68.5
diamide	PDA		15.6	86.2	20.3
diamide	PDA	NEM	15.9	74.2	
diamide	diamide		15.1	84.2	51.6
diamide	NEM		13.4	46.9	26.2
diamide	iodoacetamide		11.0	22.9	21.2

[a] RBC (2×10^8/mL) were incubated with the primary inhibitor, washed, and treated sequentially with the indicated secondary and tertiary inhibitors using the concentrations indicated in Table I. After a final wash, the cells' ability to transport NBD-PS was determined as described under Experimental Procedures. The values are from a representative experiment repeated 5–10 times. [b] NT, no treatment.

involved in the transbilayer movement of NBD-PS, it should also be possible to distinguish between them by differential sensitivity to reduction. The results shown in Figure 2 indicate that diamide-induced inhibition of NBD-PS transport was about 5 times more sensitive to cysteamine than was the inhibition induced by PDA. Differential reduction could not be attained with DTT or mercaptoethanol (not shown).

Due to the ability of cysteamine to differentiate between diamide- and PDA-induced inhibition, it became possible to determine whether sequential inhibitor treatment would block the sites of inhibition affected by a second and third inhibitor. To explore this possibility, RBC were sequentially treated with diamide, PDA, and NEM. In contrast to the results obtained with diamide treatment alone, NBD-PS transport was not reversible with cysteamine after treatment of the cells with diamide followed by PDA (Table II). Thus, in agreement with the inability of diamide to inhibit labeling of the 32-kDa polypeptide with ^{125}I-PDA (Figure 1), diamide did not protect against PDA-mediated inhibition of NBD-PS transport nor did it protect against NEM-induced inhibition. On the other hand, treatment with diamide followed by PDA did protect against NEM. This can also be seen from the observation that cysteamine (which selectively reduces only diamide-induced oxidation) did not reverse the effects of NEM on diamide-treated cells, whereas pretreatment of RBC with PDA significantly inhibited the action of NEM. These results suggest that at least two distinct sulfhydryl sites, which can be differentially oxidized by PDA and diamide and selectively reduced with cysteamine, are involved in the transport of PS and that both sites must be reduced for transport to proceed.

Diamide-Mediated Inhibition of NBD-PS Transport Is Reversible by Endogenous Glutathione. Since diamide cross-links spectrin and depletes intracellular glutathione (Haest et al., 1977), we reasoned that if the site of diamide inhibition is on an endofacial protein, regeneration of reduced glutathione to physiological levels would concomitantly reduce those sites cross-linked by diamide and subsequently result in the reappearance of normal NBD-PS transport. The results presented in Figure 3 and Table III show that incubation of diamide-treated (Figure 3A) and PDA-treated (Figure 3b)

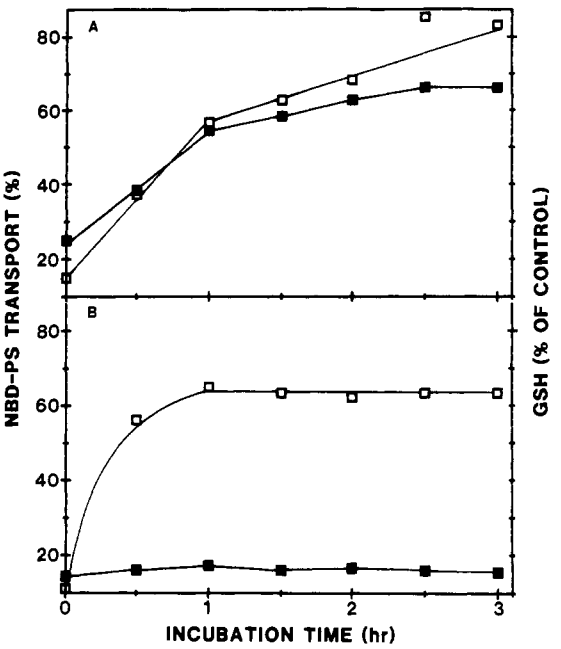

FIGURE 3: Kinetics of regeneration of GSH and NBD-PS transport. RBC were pretreated with diamide (A) or PDA (B) as described in Table I. The cells were then washed and incubated for the indicated times at 37 °C, after which their GSH content and ability to transport NBD-PS were determined. (□) GSH; (■) NBD-PS transport.

Table III: Effect of Sulfhydryl-Reactive Reagents on Cellular GSH, Membrane Sulfhydryls, and ATP[a]

treatment	SH and ATP (nmol/10^8 RBC) GSH 0 h	GSH 2 h	membrane sulfhydryls 0 h	membrane sulfhydryls 2 h	ATP 2 h
control	13.7	12.8	129	107	9.9
NEM	0	0	73	75	9.3
iodoacetamide	0	0	83	79	2.2
PDA	0.9	8.6	70	118	10.0
diamide	3.7	11.2	91	125	8.8
DTNB	12.9	12.9			7.5
azide/deoxyglucose	10.2				0.3

[a] RBC were treated with the indicated reagents as described in Table I. The cells were then washed and assessed for GSH, membrane sulfhydryls, and ATP immediately or after an additional incubation at 37 °C for 2 h. All buffers contained glucose, with the exception of azide/deoxyglucose. The values are from a representative experiment repeated 5–10 times.

RBC at 37 °C resulted in the regeneration of GSH and membrane protein sulfhydryls to near normal levels within a 30-min to 2-h period. These treatments did not affect ATP levels. In contrast to PDA-treated cells, diamide-treated cells displayed a concomitant reversal in inhibition of NBD-PS transport (Figure 3A), the kinetics of which directly coincided with regeneration of intracellular GSH. The observed increase in NBD-PS transport in the diamide-treated cells can, most probably, be attributed to GSH-mediated reduction of an oxidized membrane sulfhydryl that resides on the endofacial surface.

In an effort to determine if protein cross-linking is responsible for diamide-mediated inhibition of PS transport, ghosts were prepared from inhibitor-treated RBC, and membrane proteins were analyzed by SDS–PAGE (Figure 4). It can be seen that diamide treatment resulted in the appearance of several high molecular bands [presumably cross-linked spectrin (Haest et al., 1977)]. These high molecular weight bands disappeared after the cells were incubated at 37 °C in the presence of DTT or cysteamine; this disappearance coincides

Table IV: Effect of Glucose and Reducing Reagents on DTNB-Mediated Inhibition of NBD-PS Transport[a]

	control cells			DTNB-treated cells		
	ATP[b]	GSH[b]	NBD-PS transport (%)	ATP	GSH	NBD-PS transport (%)
control RBC[c]	9.5	13.7	80.0			
control[d]	3.4	14.5	61.7	3.6	3.1	24.1
glucose[e]	9.6	15.0	69.4	9.2	13.9	66.6
DTT[e]	2.8	20.4	62.2	2.7	20.4	57.0
cysteamine[e]	3.5	16.2	56.3	3.3	4.0	47.7
GSH[e]					3.6	25.9
cysteamine + glucose[f]					13.5	60.0
GSH + glucose[f]					4.9	31.9
glucose alone[f]					4.4	36.7

[a] RBC were washed 4 times with glucose-free PBS at 4 °C. The cells were then incubated at 37 °C for 3 h in the presence or absence of DTNB (5 mM). DTT, cysteamine, and GSH were added to the cells just prior to the addition of NBD-PS (see Table I). Glutathione and ATP levels were assessed after incubation of the cells with the reducing reagents for 45 min at 37 °C. The values are from a representative experiment repeated 5–10 times. [b] Nanomoles per 10^8 RBC. [c] Control RBC were maintained in the presence of glucose at all times. [d] Cells incubated at 37 °C without glucose. [e] For the glucose-treated cells, glucose was added to the cells after 2 h (total incubation time with glucose for 1 h), washed with glucose-free PBS, and analyzed for their ability to transport NBD-PS in the absence of additional glucose. The concentrations of glucose, DTT, cysteamine, and GSH were 10, 5, 2.5, and 10 mM, respectively. [f] Cysteamine (2.5 mM) and GSH (10 mM) were added together with glucose (10 mM) for 45 min at 37 °C.

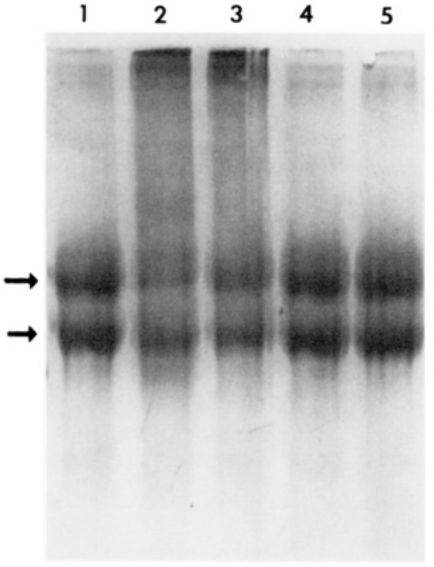

FIGURE 4: Reversibility of diamide-induced cross-linking. RBC were treated with 5 mM diamide for 30 min at 20 °C. The cells were then washed and incubated at 37 °C for 45 min in the absence or presence of DTT (5 mM) or cysteamine (2.5 mM). After the cells were washed, ghosts were prepared with 5 mM phosphate buffer (pH 8) and electrophoresed (in the absence of reducing reagents) in 5% gels. Lane 1, control RBC; lane 2, RBC immediatley after diamide treatment; lanes 3, 4, and 5, diamide-treated RBC incubated for 45 min with PBS, DTT, and cysteamine, respectively. Arrows denote spectrin bands.

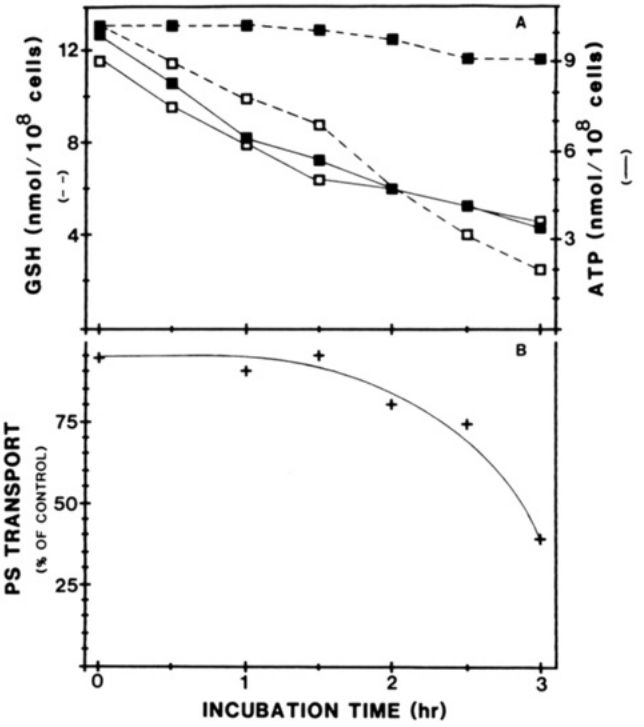

FIGURE 5: Inhibition of NBD-PS transport by transmembrane induction of GSH depletion. RBC were incubated for the indicated times in the presence or absence of 5 mM DTNB in glucose-free PBS. At various intervals, aliquots of cells were assessed for their ATP and GSH levels (A) and for their ability to transport NBD-PS (B). (Panel A) (■) Cells incubated without DTNB; (□) cells incubated with DTNB. (Panel B) Transport of NBD-PS. The data presented are expressed as the ratio of transport obtained from RBC incubated in the presence of DTNB to that obtained in the absence of DTNB to negate the effect of partial ATP depletion.

with the regeneration of GSH and return in transport activity (Figure 3).

Use of Intracellular Glutathione as an Endogenous Inhibitor of NBD-PS Transport. In the experiments described up to this point, inhibition of transport by diamide coincided with both cross-linking of spectrin and depletion of reduced intracellular GSH. One interpretation of this finding is that there are sulfhydryls on spectrin (or other endofacial proteins) that must be in the reduced state of facilitate transport of NBD-PS. Upon regeneration of intracellular GSH levels to normal, the cross-linked spectrin (oxidized sulfhydryls) is reduced back to its functional state.

To examine the possible role of GSH in maintaining the required oxidative status for transport activity, intracellular GSH was oxidized to form mixed disulfides with endofacial proteins. This was accomplished by the transmembrane response of RBC to oxidation of surface sulfhydryls by DTNB as described by Reglinski et al. (1988). Figure 5A shows that incubation of RBC with DTNB in the absence of glucose results in a time-dependent decrease in the amount of available intracellular GSH. Due to the absence of glucose (which is required for GSH depletion in this system), there was also a time-dependent decrease in cellular ATP levels independent of the presence of DTNB. Once intracellular GSH levels were depleted below a threshold, however, significant decreases in NBD-PS transport occurred (Figure 5B). It should be noted that the decrease in ATP in the control cells reduced their ability to transport NBD-PS by ~20%, whereas the DTNB-treated cells displayed >70% inhibition. Inhibition in NBD-PS transport could be reversed by the addition of glucose, DTT, or cysteamine but not by exogenously added GSH (Table IV). Although the addition of glucose resulted in the most dramatic increase in transport activity (probably because of the com-

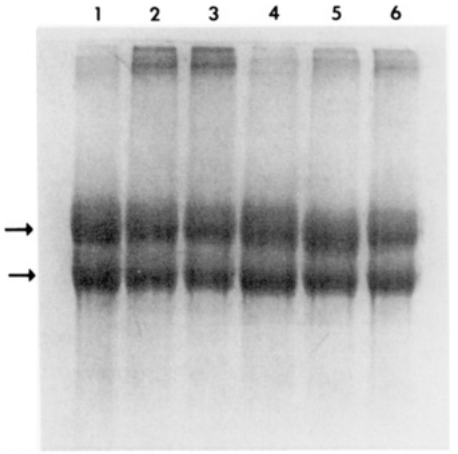

FIGURE 6: Reversibility of DTNB-induced cross-linking. RBC were incubated with DTNB (5 mM for 3 h at 37 °C in the absence of glucose). The cells were washed and incubated at 37 °C for 45 min in the absence or presence of DTT (5 mM) or cysteamine (2.5 mM). Ghosts were prepared from washed cells with 5 mM phosphate buffer (pH 8) and electrophoresed (in the absence of reducing reagents) in 5% gels. Lane 1, control RBC; lane 2, RBC immediately after treatment with DTNB; lanes 3, 4, and 5, DTNB-treated RBC incubated for 45 min with PBS, DTT, and cysteamine, respectively; lane 6, RBC incubated in the presence of glucose (10 mM) for 2 h at 37 °C. Arrows denote spectrin bands.

bination of increased ATP and GSH levels), all of the treatments, with the exception of cysteamine, resulted in the reappearance of GSH above the threshold required for normal transport activity.

Analysis of DTNB-treated RBC by SDS–PAGE revealed that this treatment (as well as diamide treatment; see Figure 4) resulted in the generation of high molecular weight complexes (Figure 6). The addition of glucose and DTT, which induces the regeneration of GSH (Table IV), resulted in the concomitant disappearance of these high molecular weight complexes. Interestingly, cysteamine, which, in the absence of glucose, does not result in the reappearance of GSH (Table IV), was nonetheless capable of reducing the high moelcular weight species, resulting in increased transport activity (see Discussion).

Lipid Asymmetry Is Stable in Cells after Having Been Established. The results presented above demonstrate that the transport of PS requires cooperation between two independent sites, a critical sulfhydryl on the 32-kDa polypeptide and another on an endofacial protein. With this information and the availability of methods to selectively oxidize either of the sites, we were able to determine whether oxidation would facilitate the inside to outside translocation of asymmetrically (inside only) distributed NBD-PS. The data presented in Table V show that conditions (oxidation of either sulfhydryl) could not be found that facilitated the transport of NBD-PS from the inner to outer leaflet. This observation suggests that once asymmetry of exogenously inserted PS is established, it is not reversed even upon oxidation of those sulfhydryls critical to the initial generation of asymmetry.

Table V: Inside-to-Outside Translocation of NBD-PS in RBC[a]

inhibitor	% of NBD-PS in outer leaflet[a]	inhibitor	% of NBD-PS in outer leaflet[a]
control	1.7 ± 1.5	diamide	0.3 ± 4.0
NEM	6.5 ± 2.1	DTNB[b]	2.0 ± 7.1
iodoacetamide	0.5 ± 0.5	azide/deoxyglucose	7.8 ± 2.8
PDA	4.7 ± 9.2		

[a] RBC were allowed to transport NBD-PS to their inner leaflets. Residual lipid remaining in the outer leaflets was removed by back-exchange. These cells, free of NBD-PS in their outer leaflet, were then treated with the inhibitors as described in Table I, washed, and incubated at 37 °C for 45 min. The amount of NBD-PS that translocated from the inner to outer leaflet was then determined by back-exchange at 4 °C. The values are the mean ± SD of six independent experiments. [b] RBC were incubated with 5 mM DTNB in glucose-free PBS for 3 h.

Discussion

It is well established that phospholipids are asymmetrically distributed in mammalian cell membranes (Etemadi, 1980). Although the mechanism responsible for maintaining PS on the endofacial surface is not well understood, several laboratories have observed that exogenously supplied PS rapidly and specifically translocates from its site of insertion in the outer leaflet to the inner leaflet in RBC (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985; Tilley et al., 1986) as well as in platelets (Sune et al., 1987), lymphocytes (Zachowski et al., 1987), and fibroblasts (Martin & Pagano, 1987). This phenomenon has been shown to be an active process which requires the participation of a specific enzyme (Zachowski et al., 1986).

We previously identified a 32-kDa integral membrane polypeptide that participates in the transport process and contains a hydrophobic sulfhydryl site (Schroit et al., 1987; Connor & Schroit, 1988). Oxidation of this site results in complete inhibition of PS translocation, which can be reversed upon its reduction with membrane-permeable reducing agents. In this study, we examined the transport of exogenously inserted NBD-PS in RBC and have shown that the oxidative status of at least two distinct sulfhydryl sites is critical for the transport process. These experiments were possible because cysteamine preferentially reduces sites oxidized by diamide and DTNB but not by PDA. The data indicate that the second site resides on the endofacial surface and that the roles of this site and the 32-kDa polypeptide are complementary: Both must be operative for transport to proceed.

Several lines of evidence support the contention that an endofacial protein distinct from the 32-kDa polypeptide is intimately involved in the transbilayer movement of PS. First, diamide treatment of RBC did not block the 32-kDa sulfhydryl; no differences could be detected by ^{125}I-PDA labeling, and diamide did not protect against PDA-mediated inhibition of PS transport. This is evident because cysteamine did not reverse the inhibition in NBD-PS transport after sequential treatment of RBC with diamide and PDA. Second, oxidative cross-linking and inhibition of PS transport by diamide were reversible by intracellular glutathione whereas PDA-mediated inhibition was not. Third, application of an external oxidative stimulus resulted in endogenous endofacial protein cross-links [see Reglinski et al. (1988)] that inhibited translocation. It appears that the mechanism responsible for the inhibition of NBD-PS transport by DTNB is similar to diamide since the effects of both were reversible either directly by cysteamine or by the regeneration of GSH. Furthermore, DTNB-mediated endofacial protein cross-links were independent of the 32-kDa polypeptide as shown by its ability still to be labeled with ^{125}I-PDA (data not shown). At this time, it is not clear why the addition of cysteamine does not result in the regeneration of GSH. Related experiments have indicated that GSH (which appears to be in its oxidized, GSSG form)[2] is regenerated if glucose is added together with the cysteamine

[2] Preliminary data suggest that most of the DTNB-mediated depletion of intracellular GSH is due to the formation of GSSG and not mixed GSH–endofacial protein disulfides.

for the 45-min incubation. The addition of glucose alone for 45 min, however, is not enough to regenerate GSH to levels sufficient to reduce protein cross-links or induce increased transport activity (see Table IV). Identical results were obtained (diminished high molecular weight cross-links, no generation of GSH, and increased NBD-PS transport) upon the addition of cysteamine to cells incubated with diamide in the absence of glucose. Whatever the mechanism, increased NBD-PS transport is probably due to the reduction of cross-linked protein and not to the presence or absence of GSH per se. Indeed, RBC ghosts (which do not contain GSH) prepared in the presence of ATP retain full transport activity (Seigneuret & Devaux, 1984).

Oxidation of either the PDA-sensitive or the diamide/DTNB-sensitive sites did not promote the movement of NBD-PS from the inner to outer leaflet. This suggests that once PS is localized in the inner leaflet, it is stabilized and can no longer be translocated regardless of the oxidative status of the cell. This finding is inconsistent with interpretations of experimental data suggesting that an energy (ATP)-dependent outward movement of PS occurs in RBC (Bitbol & Devaux, 1988; Sune et al., 1988) and that cytoskeletal proteins are not involved in the transport of exogenously added PS analogues from the outer to inner leaflet (Calvez et al., 1988). Our inability to detect large amounts of transport from the inner to outer leaflet was not due to lack of ATP since none of the sulfhydryl reagents (with the exception of iodoacetamide) induce ATP depletion. It should be noted, however, that we cannot be certain whether the 5–10% of the NBD-PS that was removed from the RBC with acceptor vesicles after back-exchange was not from the inner leaflet. However, if flop were significant, one would expect that long-term incubation of RBC with acceptor vesicles at 37 °C would force a shift in the equilibrium distribution of PS from the RBC to the acceptor membranes. Incubations of up to 3 h with acceptor membranes, irrespective of the inclusion of the sulfhydryl oxidants, did not alter the results.

Our results suggest that cytoskeletal proteins play a crucial role in the maintenance of PS asymmetry. Although one might argue that a diamide/DTNB-sensitive site could be present on the cytoplasmic portion of the 32-kDa polypeptide, this would seem unlikely because diamide- or DTNB-induced cross-linking of endofacial proteins did not result in detectable molecular weight shifts in ^{125}I-PDA-labeled 32-kDa polypeptide.

Spectrin (Haest, 1982; Cohen et al., 1986; Maksymiw et al., 1987) and protein band 4.1 (Sato & Ohnishi, 1983; Rybicki et al., 1988; Cohen et al., 1988; Shiffer et al., 1988) seem to be good candidates for the endofacial component sensitive to mixed-disulfide formation with diamide or DTNB since both have been shown to bind PS. These proteins could represent a component essential to the maintenance of PS asymmetry. Clearly, more experimentation is needed to delineate their possible roles in the transport process.

Acknowledgments

We thank Dr. Philip Frost for his helpful discussions and Karen Gillum, Barbara Hunt, and Kenneth Dunner, Jr., for technical assistance.

Registry No. GSH, 70-18-8; 5′-ATP, 56-65-5; glucose, 50-99-7.

References

Beutler, E., Duron, O., & Kelly, B. M. (1963) *J. Lab. Clin. Med. 61*, 882–888.

Bitbol, M., & Devaux, P. F. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6783–6787.

Calvez, J.-Y., Zachowski, A., Herrmann, A., Morrot, G., & Devaux, P. F. (1988) *Biochemistry 27*, 5666–5670.

Cohen, A. M., Liu, S. C., Derick, L. H., & Palek, J. (1986) *Blood 68*, 920–926.

Cohen, A. M., Liu, S. C., Lawler, J., Derick, L., & Palek, J. (1988) *Biochemistry 27*, 614–619.

Comfurius, P., & Zwaal, R. F. A. (1977) *Biochim. Biophys. Acta 488*, 36–42.

Connor, J., & Schroit, A. J. (1987) *Biochemistry 26*, 5099–5105.

Connor, J., & Schroit, A. J. (1988) *Biochemistry 27*, 848–851.

Daleke, D. L., & Huestis, W. H. (1985) *Biochemistry 24*, 5406–5416.

Dressler, V., Haest, C. W. M., Plasa, G., Deuticke, B., & Erusalimsky, J. D. (1984) *Biochim. Biophys. Acta 775*, 189–196.

Etemadi, A.-H. (1980) *Biochim. Biophys. Acta 604*, 423–475.

Gordesky, S. E., Marinetti, G. V., & Love, R. (1975) *J. Membr. Biol. 20*, 111–132.

Habeeb, A. F. S. A. (1972) *Methods Enzymol. 25*, 457–464.

Haest, C. W. M. (1982) *Biochim. Biophys. Acta 694*, 331–352.

Haest, C. W. M., & Deuticke, B. (1976) *Biochim. Biophys. Acta 436*, 353–365.

Haest, C. W. M., Kamp, D., Plasa, G., & Deuticke, B. (1977) *Biochim. Biophys. Acta 469*, 226–230.

Haest, C. W. M., Plasa, G., Kamp, D., & Deuticke, B. (1978) *Biochim. Biophys. Acta 509*, 21–32.

Laemmli, U. K. (1970) *Nature (London) 16*, 680–685.

Maksymiw, R., Sui, S.-F., Gaub, H., & Sackmann, E. (1987) *Biochemistry 26*, 2983–2990.

Martin, O., & Pagano, R. E. (1987) *J. Biol. Chem. 262*, 5890–5898.

Op den Kamp, J. A. F. (1979) *Annu. Rev. Biochem. 48*, 47–71.

Reglinski, J., Hoey, S., Smith, E. W., & Sturrock, R. D. (1988) *J. Biol. Chem. 263*, 12360–12366.

Rothman, J. E., & Lenard, J. (1977) *Science 195*, 743–753.

Rybicki, A. C., Heath, R., Lubin, B., & Schwartz, R. S. (1988) *J. Clin. Invest. 81*, 255–260.

Sato, S., & Ohnishi, S. (1983) *Eur. J. Biochem. 130*, 19–25.

Schroit, A. J., Madsen, J., & Ruoho, A. E. (1987) *Biochemistry 26*, 1812–1819.

Seigneuret, M., & Devaux, P. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3751–3755.

Shiffer, K. A., Goerke, J., Duzgunes, N., Fedor, J., & Shohet, S. B. (1988) *Biochim. Biophys. Acta 937*, 269–280.

Steck, T. L. (1974) *Methods Membr. Biol. 2*, 245–281.

Sune, A., Bette-Bobillo, P., Bienvenue, A., Fellmann, P., & Devaux, P. F. (1987) *Biochemistry 26*, 2972–2978.

Sune, A., Vidal, M., Morin, P., Sainte-Marie, J., & Bienvenue, A. (1988) *Biochim. Biophys. Acta 946*, 315–327.

Tilley, L., Cribier, S., Roelofsen, B., Op den Kamp, J. A. F., & van Deenen, L. L. M. (1986) *FEBS Lett. 194*, 21–27.

Verkleij, A. J., Zwaal, R. F. A., Roelofsen, B., Comfurius, P., Kastelijn, D., & van Deenen, L. L. M. (1973) *Biochim. Biophys. Acta 323*, 178–193.

Williamson, P., Antia, R., & Schlegel, R. A. (1987) *FEBS Lett. 219*, 316–320.

Zachowski, A., Favre, E., Cribier, S., Herve, P., & Devaux, P. F. (1986) *Biochemistry 25*, 2585–2590.

Zachowski, A., Herrmann, A., Paraf, A., & Devaux, P. F. (1987) *Biochim. Biophys. Acta 897*, 197–200.