Inhibition and Stimulation of Phospholipid Scrambling Activity. Consequences for Lipid Asymmetry, Echinocytosis, and Microvesiculation of Erythrocytes

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Received April 12, 2001; Revised Manuscript Received June 11, 2001

ABSTRACT: An increase of the intracellular Ca^{2+} concentration in erythrocytes is known to activate rapid nonspecific bidirectional translocation of membrane-inserted phospholipid probes and to decrease the asymmetric distribution of endogenous membrane phospholipids. These scrambling effects are now shown to be suppressed by pretreatment of cells with the essentially impermeable reagents 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid and 2,4,6-trinitrobenzenesulfonate. The inhibitory effects are no longer observed during renewed activation of scrambling following a first transient activation by Ca^{2+}. Assuming the involvement of the human scramblase, this suggests a conformational alteration of this protein during activation by Ca^{2+}. Marked suppression of scrambling activity is also observed in cells pretreated with the disulfide reducing agent dithioerythritol which can be reverted by the SH oxidizing agent diamide. This indicates the importance of intramolecular and/or intersubunit disulfide bonds for the function of the scramblase. On the other hand, treatment of cells with the SH reagents *N*-ethylmaleimide and phenylarsine oxide enhances Ca^{2+}-activated scrambling and diminution of asymmetry of membrane phospholipids. This suggests an allosteric connection of several protein SH groups to the translocation mechanism. The inhibitors retain their strong suppressive effects. Besides covalent modification, addition of oligomycin highly stimulates and addition of clotrimazole suppresses the Ca^{2+}-activated translocation. No evidence for a role of the ATP-binding cassette transporter ABCA1 in the Ca^{2+}-activated outward translocation is obtained. Suppression of phospholipid scrambling by dithioerythritol inhibits Ca^{2+}-induced spheroechinocytosis and reduces the extent of subsequent microvesiculation. Scrambling of endogenous phospholipids is proposed to induce echinocytosis and to have only a stimulatory effect on microvesiculation.

The asymmetric distribution of phospholipids in cell membranes of platelets, erythrocytes, and other cells is rapidly lost upon an increase of the intracellular Ca^{2+} concentration (*1*−*4*). This loss results from the activation of a translocation system for endogenous phospholipids which strongly enhances bidirectional transbilayer movement of all endogenous membrane phospholipid species (*5, 6*) as well as of lipid probes which are inserted into the membrane (*3, 7*). This so-called scrambling takes place during cell activation (*1, 8*) and apoptosis (9−*11*) and results in the exposure of the inner membrane leaflet phospholipid, phosphatidylserine, to the outer leaflet. This exposure is an important signal for removal of cells by phagocytes (*12*−*16*). The concentration of free intracellular Ca^{2+} for half-maximal activation of scrambling is 25−100 μmol L^{-1} (*11, 17*−*19*). Recently, a membrane protein with characteristics of a scramblase has been cloned (*20*−*22*). It is a protein of 318 amino acid residues in human erythrocytes with a single transmembrane domain, a potential Ca^{2+}-binding EF hand motif adjacent to this membrane domain, and potential interaction sites for WW or SH3 domains of other proteins (*21, 22*), as well as a potential protein kinase C phosphorylation site involved in regulation (*23*). Acylation of one or more of the SH groups of cysteine residues of the scramblase is required for its function (*24*). Thioester-linked acyl chains have been proposed to promote the correct conformation for the Ca^{2+}-binding site or to participate in acceleration of transbilayer movement of lipids (*24*).

Although expression of phospholipid scramblase in various human cell lines correlates with the level of Ca^{2+}-induced exposure of phosphatidylserine to the outer membrane surface (*25*), other factors may play a role in scrambling (*26*−*29*). Intracellular polyamines or analogous motifs on proteins have been suggested to induce phospholipid scrambling by formation of domains of phosphatidylinositol-4,5-bisphosphate (*27*). Recently, intracellular polyamines have been found to modulate Ca^{2+}-induced flip-flop (*28*). Moreover, the ATP-binding casette (ABC)[1] transporter type A1 has been reported to promote Ca^{2+}-activated externalization of phosphatidylserine (*30, 31*). It has been suggested either that

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[1] Abbreviations: ABC, ATP-binding cassette; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonic acid; DTE, dithioerythritol; MMI, mean morphological index; NBD-PC, 1-oleoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl]-*sn*-glycero-3-phosphocholine; NBD-PS, 1-oleoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl]-*sn*-glycero-3-phosphoserine; NBD-SM, 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl]sphingosylphosphocholine; NDP1, 10-(naphth-1-yl)-1-decylphosphoric acid; NDP2, 10-(naphth-1-yl)-1-decanephosphonic acid; NEM, *N*-ethylmaleimide; PAO, phenylarsine oxide; TNBS, 2,4,6-trinitrobenzenesulfonate.

10.1021/bi0107492 CCC: $20.00
Published on Web 07/12/2001

ABCA1 behaves as a transporter which exclusively translocates phosphatidylserine to the outer leaflet or that it is part of a complex that controls transbilayer organization of phospholipids (*31*).

In the present study, effects of covalent chemical membrane modification on the Ca^{2+}-activated enhancement of transmembrane movement of phospholipid probes and scrambling of membrane phospholipids as well as resulting effects on cell shape and membrane microvesiculation are presented. Ca^{2+}-activated scrambling will be shown to become enhanced by modification of protein SH groups and suppressed by the disulfide reducing agent dithioerythritol as well as the covalent inhibitor of anion transport 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS) and the amino reagent 2,4,6-trinitrobenzoate (TNBS). Ca^{2+}-activated scrambling is also shown to be altered by noncovalent modifying agents. Changes in the extent of scrambling of membrane phospholipids correlated with changes in the extent of echinocytosis and membrane microvesiculation.

EXPERIMENTAL PROCEDURES

Materials. Fresh human blood was obtained from healthy donors. Erythrocytes were isolated by centrifugation and after removal of lymphocytes washed 3 times with isotonic saline.

Clotrimazole, diamide, dithioerythritol (DTE), ionophore A23187, glibenclamide, oligomycin, verapamil, orthovanadate, *N*-ethylmaleimide (NEM), phenylarsine oxide (PAO), 2,4,6-trinitrobenzenesulfonate (TNBS), and bee venom phospholipase A_2 were obtained from Sigma; 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS) was from Pfaltz and Bauer (Waterbury, CT); and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) were from Calbiochem. MK571 was supplied by Alexis Corp.

10-(Naphth-1-yl)-1-decylphosphoric acid (NDP1) was synthesized as described before (*32*). 10-(Naphth-1-yl)-1-decanephosphonic acid (NDP2) was synthesized from 10-(naphth-1-yl)-1-decanol (*32*) and phosphotribromide to yield the intermediary compound 10-(naphth-1-yl)-1-bromodecane. Subsequently, reaction of this compound with dibutyl phosphite gave 10-(naphth-1-yl)-1-decane phosphonic acid dibutyl ester which after hydrolysis using NaJ and trimethylsilyl chloride produced NDP2.

1-Oleoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC) was obtained from Avanti Polar Lipids and 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl]sphingosylphosphocholine (NBD-SM) from Molecular Probes. 1-Oleoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl]-*sn*-glycero-3-phosphoserine (NBD-PS) was synthesized from NBD-PC (*33*).

The incubation medium (pH 7.4) contained the following (mmol L^{-1}): KCl (90), NaCl (45), phosphate (12.5), and sucrose (44) (=KNPS) or dextran 4 (9 g dL^{-1}) (=KNPD). The latter additives protected the cells against colloid-osmotic hemolysis. In experiments with Ca^{2+}, phosphate was exchanged for HEPES (10 mmol L^{-1}) with the corresponding abbreviation: KNHS. Routinely, measurements of microvesiculation were carried out in a medium containing the following (mmol L^{-1}): NaCl (150), HEPES (10), and sucrose (44) (=NHS).

Pretreatment of Erythrocytes. Covalent Modification of Erythrocytes. Modifications with SH reagents were carried out in KNPS (10% hematocrit, 37 °C) containing either 0.8 mmol L^{-1} NEM, 0.2−1 mmol L^{-1} PAO (both 20 min); 0.5−5 mmol L^{-1} diamide, 5 $mmol^{-1}$ L NEM (both 30 min); or 10 mmol L^{-1} DTE (45 min) followed by three washings of the cells with KNPS.

Modifications with covalent inhibitors of anion exchange were carried out in KNPS (10% hematocrit) containing 50 μmol L^{-1} DIDS or 2 mmol L^{-1} TNBS (30 min, 37 °C), followed by three washings of the cells in KNPS. In some cases, as indicated in the text, reagent concentrations and incubation times were varied.

Preincubation with Ca^{2+}/A23187. Erythrocytes were incubated in KNHS containing 0.1−0.5 mmol L^{-1} Ca^{2+} and 10 μmol L^{-1} A23187 (0−60 min, 37 °C) and washed 2 times with KNHS containing 5 mmol L^{-1} EGTA, followed by 2 washings with KNHS containing 1 g dL^{-1} albumin and 3 washings with KNHS.

Measurement of the Inward Translocation (Flip) of Fluorescent Lipids. After insertion of trace amounts of fluorescent NBD-phospholipid (8 nmol/mL of packed cells) or NDP1 or NDP2 (200 nmol/mL of packed cells) into the outer membrane leaflet of erythrocytes, cells were washed once and resuspended in KNHD (12% hematocrit, 0 °C) containing 0−0.5 mmol L^{-1} Ca^{2+}. After addition of 10 μmol L^{-1} ionophore A23187, the suspension was incubated at 37 °C, and the time-dependent flip of the probes to the inner membrane leaflet was measured by following the increase of fluorescence in the inner membrane leaflet using the albumin extraction procedure. First-order rate constants for the flip process were derived from the data by computer fitting as described before (*32, 33*). Hemolysis after 120 min of flip was <2%.

Measurement of the Outward Translocation (Flop) of NBD-Labeled Phosphatidylserine. After loading the inner membrane leaflet with NBD-PS by an initial flip period (45 min, 37 °C), residual probe in the outer leaflet was removed by albumin and the flop of the probe from the inner to the outer leaflet followed by measuring the time-dependent decrease of fluorescence in the inner leaflet using the albumin extraction procedure as described (*33*).

Measurement of Transbilayer Distribution of Membrane Phospholipids. Cells that had been pretreated with Ca^{2+} and ionophore were washed twice with KNHS containing 5 mmol L^{-1} EDTA to remove Ca^{2+} from the cells, twice with KNHS containing 1 g dL^{-1} albumin to extract the ionophore, and 3 times with KNHD to remove albumin. Native and pretreated cells were then exposed to phopholipase A_2 from bee venom (3 units/mL medium) in KNHD containing 0.25 mmol L^{-1} Ca^{2+} and Mg^{2+}. After incubation (10 min at 37 °C), phospholipase activity was blocked by addition of 1 mmol L^{-1} EDTA, lipids were extracted from the cells, and the extent of cleavage of various phospholipids was determined as described before (*34*). Hemolysis was <2%.

Microscopy. The morphology of erythrocytes in KNHS containing 0.5 mmol L^{-1} Ca^{2+} and 10 μmol L^{-1} ionophore was assessed by interference contrast microscopy (1000× magnification) following fixation with glutardialdehyde. A morphological score (MI) was assigned to each of up to 100 cells and the mean morphological index (MMI) calculated as described before (*34*).

Measurement of Microvesiculation. NBD-SM was inserted into the outer membrane leaflet of native and pretreated

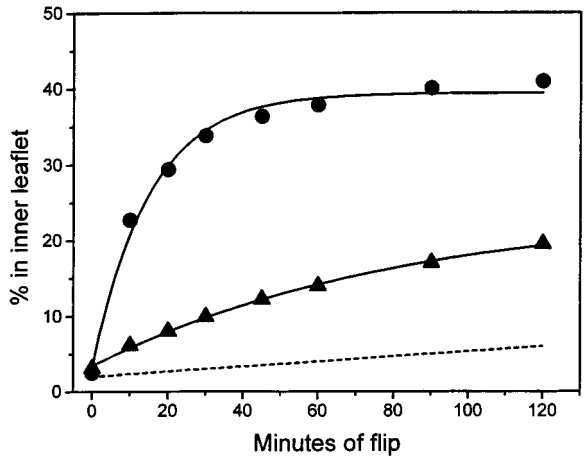

FIGURE 1: Activation of flip of NBD-PC by an increase of intracellular Ca^{2+} is suppressed by DIDS. Erythrocytes were treated (30 min, 37 °C) with 50 μmol L^{-1} DIDS. After insertion of NBD-PC into the outer membrane leaflet of control (●) and DIDS-treated (▲) cells, the flip of the probe to the inner leaflet in the presence of 0.5 mmol L^{-1} Ca^{2+} and 10 μmol L^{-1} A23187 was measured (see Experimental Procedures). The dashed line represents the slow flip in the absence of Ca^{2+}.

erythrocytes, or NBD-PS was allowed to translocate to the inner membrane leaflet and then removed from the outer leaflet. Cells labeled with either of the two NBD-phospholipids were incubated at 37 °C in NHS or KNHS (10% hematocrit) containing 0.5 mmol L^{-1} Ca^{2+} and 10 μmol L^{-1} A23187. At variable time intervals, samples were taken and cells sedimented by centrifugation (20 s, 10000*g*). The fluorescence of the supernatant originating from nonsedimented membrane vesicles was then measured and related to the total fluorescence in the original suspension. Hemolysis of Ca^{2+}-loaded erythrocytes quantified by the hemoglobin content of the supernatant after 30 min of incubation was <2%. The extent of shrinkage of cells due to salt loss following activation of the Ca^{2+}-activated K^+ channel was derived from the time-dependent decrease of the hematocrit of the cell suspensions.

RESULTS

Ca^{2+}-Activated Translocation of NBD-PC from the Outer to the Inner Membrane Leaflet (Flip). The slow flip of NBD-PC in the erythrocyte membrane is highly enhanced (up to almost 100-fold) after an increase of the intracellular Ca^{2+} concentration (Figure 1). Incubation of cells in medium containing 0.5 mmol L^{-1} Ca^{2+} and ionophore increases the flip rate constant from 0.0003 min^{-1} in control cells (*35*) to 0.0221 ± 0.0087 min^{-1} (Table 1). Under these conditions, optimal translocation rates were obtained. First, ionophore concentrations of 5−20 μmol L^{-1} produced similar kinetics. Second, an increase of the Ca^{2+} concentration to 1 mmol L^{-1} at 10 μmol L^{-1} ionophore did not significantly increase the stimulatory effect while at 2 mmol L^{-1} kinetics tend to decrease again (data not shown, ref *4*).

Pretreatment of cells with DIDS (30 min, 37 °C), well-known as a membrane-impermeable inhibitor of anion exchange (*36*), suppresses the Ca^{2+}-activated flip up to about 90% (Figure 1, Table 1). Suppression of Ca^{2+}-activated flip is observed at concentrations of DIDS considerably higher than those required to block anion exchange via AE1. This becomes clearly evident from the data shown in Figure 2.

Table 1: Accelerating and Suppressive Effects of Covalent Modifiers on the Ca^{2+}-Activated Flip of NBD-Phospholipids[a]

accelerator (mmol L^{-1})	probe	$k_{in} \times 10^2$ (min^{-1}) no inhibitor	% inhibition of flip by DIDS	TNBS	DTE
none	PC	2.21 ± 0.87 (19)	92 ± 3 (7)	90 ± 5 (6)	90 (3)
NEM (0.8)	PC	5.87 ± 1.82 (3)	88 ± 1 (3)	78 (2)	96 (2)
PAO (0.2)	PC	7.04 ± 0.64 (3)	88 ± 1 (3)	72 (2)	97 (2)
none	SM	0.25 ± 0.11 (7)			
PAO (0.2)	SM	1.00 (1)			
PAO (1)	SM	1.90 (2)			
NEM (0.8)	SM	1.02 ± 0.16 (3)			
NEM (5)	SM	2.53 ± 0.42 (4)	64 (2)		88 (2)

[a] Erythrocytes were pretreated at 37 °C with 0.8 mmol L^{-1} NEM or 0.2−1 mmol L^{-1} PAO (20 min), 5 mmol L^{-1} NEM (30 min), or 10 mmol L^{-1} DTE (45 min). After 3 washings, controls and modified cells were treated with 50 μmol L^{-1} DIDS or 2 mmol L^{-1} TNBS (30 min, 37 °C). After 3 washings and loading of cells with NBD-PC or NBD-SM, flips were measured in KNHD containing 0.5 mmol L^{-1} Ca^{2+} and 10 μmol L^{-1} A23187. Data represent mean values ± SD with the number of experiments given in parentheses.

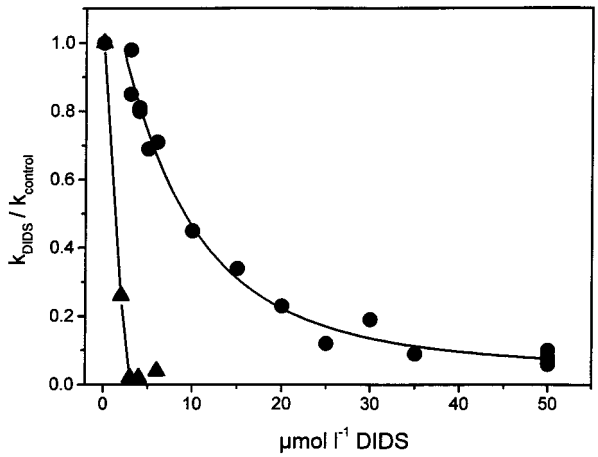

FIGURE 2: Inhibitory effect of DIDS on the Ca^{2+}-activated flip requires higher concentrations than those required for suppresssion of the flip via the anion exchanger AE1. Erythrocytes were pretreated with 0−50 μmol L^{-1} DIDS, and the flip of the anionic probe NDP1 (▲) via AE1 (in the absence of Ca^{2+}/ionophore) and the flip of NBD-PC (●) in the presence of 0.5 mmol L^{-1} Ca^{2+} and 10 μmol L^{-1} ionophore were then measured. $k_{DIDS}/k_{control}$ represents the ratio of the flip rate constant for DIDS-pretreated cells and that for untreated control cells.

Pretreatment of cells at 10% hematocrit with 3 μmol L^{-1} DIDS (30 min, 37 °C), which corresponds closely to the theoretical amount of DIDS required to completely block AE1 (*36*), highly suppresses (95%) translocation, by flip via AE1 (*32*), of the anionic naphthyl-decyl phosphate probe NDP1. In a parallel experiment, essentially no inhibition (<5%) of the Ca^{2+}-activated flip of NBD-PC is observed at this low DIDS concentration. However, an inhibition of about 40% is obtained at 10 μmol L^{-1} DIDS, increasing to about 90% at 50 μmol L^{-1} (Figure 2, Table 1). In contrast to anion exchange via AE1, the Ca^{2+}-activated flip is not inhibited when DIDS (50−500 μmol L^{-1}) is only present during flip measurement (data not shown). Besides DIDS, pretreatment of cells with the amino reagent 2,4,6-trinitrobenzenesulfonate (TNBS) at 2 mmol L^{-1} suppresses the Ca^{2+}-activated flip by about 88% (Table 1).

The Ca^{2+}-activated flip is also inhibited in the presence of the disulfide-reducing dithiol DTE during the measurement or after a pretreatment of cells with the reagent (Figure 3).

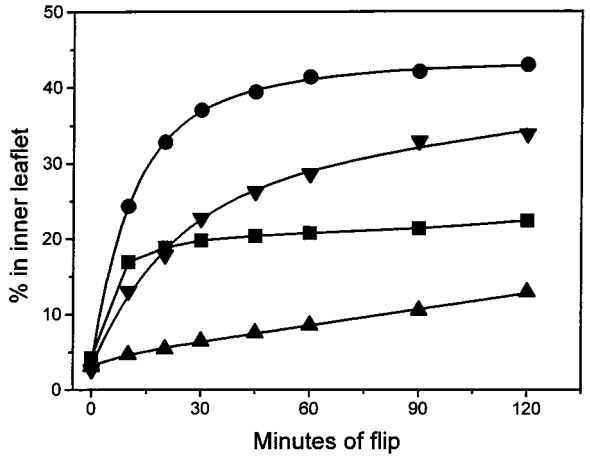

FIGURE 3: Suppression of the Ca^{2+}-acitvated flip by DTE. Erythrocytes were pretreated with DTE (10 mmol L^{-1}) for 15 or 45 min. After insertion of NBD-PC into the outer membrane leaflet, the flip in control cells in the absence or presence of DTE (10 mmol L^{-1}) as well as in cells that had been treated with DTE was measured in the presence of 0.5 mmol L^{-1} Ca^{2+} and 10 μmol L^{-1} A23187. Control cells (●), cells in the presence of DTE added at $t = 0$ (■), and cells pretreated with DTE for 15 min (▼) as well as 45 min (▲).

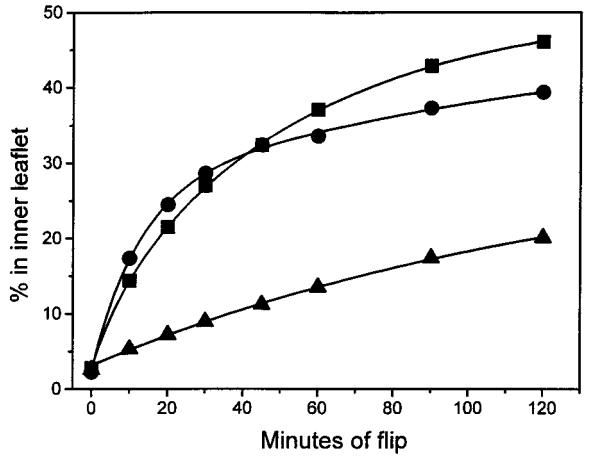

FIGURE 4: Reversal, by the SH-oxidizing agent diamide, of the DTE-induced suppression of Ca^{2+}-activated flip of NBD-PC. Control cells (●), cells treated with DTE (10 mmol L^{-1}, 45 min, 37 °C) (▲), and cells treated with DTE followed by diamide (0.5 mmol L^{-1}, 30 min, 37 °C) (■) were loaded with NBD-PC and flip rates measured in the presence of 0.5 mmol L^{-1} Ca^{2+} and 10 μmol L^{-1} ionophore.

When added during flip measurement, DTE completely suppresses the Ca^{2+}-activated flip after about 15 min (Figure 3). On the other hand, a 15 min pretreatment with the same concentration of DTE followed by removal of the thiol from the medium produced only 64% inhibition ($n = 2$). This indicates partial spontaneous restoration of the disulfide bonds in the absence of DTE. On the other hand, a 45 min pretreatment with DTE produces 90% inhibition (Table 1). The suppression of Ca^{2+}-activated flip by DTE is completely reversed by a treatment of cells with the SH-oxidizing agent diamide (Figure 4). This demonstrates the importance of disulfide bonds for Ca^{2+}-activated phospholipid flip.

In addition to the reversal of inactivation by diamide (0.5 mmol L^{-1}), this SH oxidizing agent at higher concentrations (5 mmol L^{-1}, 30 min) stimulates the Ca^{2+}-activated flip about 1.5-fold (data not shown). A higher stimulation is observed after a pretreatment of cells (20 min) with the SH alkylating reagent NEM (0.8 mmol L^{-1}) or with PAO (0.2 mmol L^{-1}), a reagent forming adducts with vicinal SH groups (*37*). These stimulations are about 3-fold (Table 1). Higher concentrations of the reagents produce still stronger effects (Table 1). Since at higher concentrations, however, the SH reagents enhance the flip of NBD-PC even in the absence of Ca^{2+}, which complicates quantitative evaluation of the Ca^{2+}-activated flip, these experiments were carried out using NBD-SM which has a 10-fold slower flip than NBD-PC (*35*). Pretreatment with 5 mmol L^{-1} NEM or 1 mmol L^{-1} PAO for 20 min produced an up to 10-fold acceleration of the Ca^{2+}-activated flip of NBD-SM (Table 1). The effect of inhibitors of Ca^{2+}-activated flip is retained after the acceleration by NEM (Table 1). In contrast to its concentration dependence, the stimulating effect of NEM and PAO did not increase significantly with the time of exposure (data not shown). These combined results may be taken as evidence that modification of several free SH groups is important for the Ca^{2+}-activated flip process. The effects of both cleavage of disulfide bonds and modification of thiol groups clearly suggest that both forms of sulfhydryls are present in the Ca^{2+}-activated protein which produces flip enhancement.

Table 2: Suppression, by a Transient Increase of the Intracellular Ca^{2+}, of the Inhibitory Effect of DIDS on a Subsequent Ca^{2+}-Activated Flip[a]

additional modification	conditions of transient Ca^{2+}-loading		inhibition of flip by DIDS (%)
	min	mmol L^{-1}	
none	60	0	~90
none	30	0.2	36
none	60	0.2	11
none	10	0.5	45
none	30	0.5	17
NEM	30	0	~90
NEM	30	0.1	17
NEM	30	0.5	7

[a] Erythrocytes that had been treated with 0.8 mmol L^{-1} NEM (20 min, 37 °C) and untreated cells were incubated (37 °C) for 30 min in media containing 0.1, 0.2, or 0.5 mmol L^{-1} Ca^{2+} and 10 μmol L^{-1} A23187. Subsequently, intracellular Ca^{2+} and ionophore were removed (see Experimental Procedures), and the cells were treated with 50 μmol L^{-1} DIDS (30 min, 37 °C). The flip rates of NBD-PC were then measured in media containing 0.5 mmol L^{-1} Ca^{2+} and ionophore. Cells not treated with DIDS served as controls. Data represent mean values of at least 2 experiments.

Interestingly, the strong suppression of Ca^{2+}-activated flip by pretreatment with DIDS (about 90%, Table 1) is strongly reduced or essentially abolished (Table 2) when the cells are DIDS-treated after a transient (10−60 min) activation by Ca^{2+} (0.2−0.5 mmol L^{-1}) followed by removal of Ca^{2+} from the cells with EGTA (see Experimental Procedures). The use of EDTA instead of EGTA did not make any difference (data not shown). Similar results were obtained for the inhibition by TNBS (data not shown). Transient exposure to Ca^{2+} after pretreatment of the cells with NEM also abolishes the inhibitory effect of DIDS (Table 2). The loss of inhibitory effects may be due to either a lack of reaction of DIDS (and TNBS) or a change of protein conformation by transient Ca^{2+} loading which precludes the inhibitory change of conformation produced by the still occurring reaction of modifiers.

Besides the flip of phospholipid probes, Ca^{2+} also enhanced the residual flip of the naphthyl-decyl phosphate and

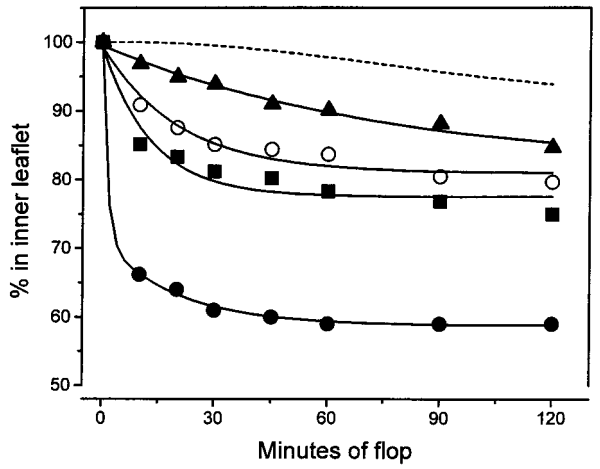

FIGURE 5: Effects of noncovalent inhibitors on Ca^{2+}-activated outward flop of NBD-labeled phosphatidylserine. The inner membrane leaflet of erythrocytes was loaded with NBD-PS and the probe extracted from the outer leaflet with albumin. Subsequently, the flop of NBD-PS was measured in the presence of vanadate to suppress active flip via the aminophospholipid flippase and active flop via MRP1. Flop in the absence of Ca^{2+} (dashed line) or in the presence of 0.5 mmol L^{-1} Ca^{2+} and 10 μmol L^{-1} A23187, without inhibitor (○), with 100 μmol L^{-1} glibenclamide (■), with 10 μmol L^{-1} oligomycin (●), or with 50 μmol L^{-1} clotrimazole (▲).

phosphonate probes (NDP1 and NDP2) after blockage of their band 3-mediated flip by pretreatment of cells with a low concentration of DIDS or flip measurements in the presence of the noncovalent inhibitor of anion exchange, DNDS (*36*). The flip rates at 25 °C for the Ca^{2+}-activated flip of NDP1 ($k_1 = 0.0113$ min^{-1}, $n = 3$), of NDP2 ($k_1 = 0.0117$ min^{-1}, $n = 5$), and of NBD-PC ($k_1 = 0.0140$ min^{-1}, $n = 3$) proved to be similar. This demonstrates the low selectivity of the Ca^{2+}-activated translocation process. As in the case of NBD-PC, Ca^{2+}-activated flips of NDP1 and NDP2 are suppressed to a high extent by pretreatment with 50 μmol L^{-1} DIDS, respectively 66% and 85% ($n = 3$), and are stimulated 2-fold by NEM (data not shown).

Ca^{2+}-Activated Translocation of NBD-PS from the Inner to the Outer Membrane Leaflet (Flop). To study the Ca^{2+}-activated flop of NBD-PS without interference by the active inward flip via the aminophospholipid flippase and the outward flop via MRP1, flop measurements were first carried out in the presence of vanadate (*35*). In line with the properties of the Ca^{2+}-activated flip, addition of DTE produces inhibition of the Ca^{2+}-activated flop, DIDS pretreatment results in inhibition, and NEM pretreatment acts stimulatory (data not shown). Glibenclamide, which is known as an inhibitor of transport via ABCA1 (*38*) and was recently found to abolish the flop via MRP1 (50 μmol L^{-1}, data not shown), does not inhibit the Ca^{2+}-activated flop of NBD-PS. At low glibenclamide concentrations (10 μmol L^{-1}), no significant effect on the flop is observed while at higher concentrations (20−200 μmol L^{-1}) even a concentration-dependent stimulation of the flop takes place (cf. Figure 5). Oligomycin, which is known as an inhibitor of the Na^+,K^+-ATPase and the Ca^{2+}-activated K^+ channel (*40*) but is also an inhibitor of transport via P-glycoprotein, MRP1 (*39*), and ABCA1 (*30*), stimulates the Ca^{2+}-activated flop. At 1 μmol L^{-1}, stimulation is already significant and becomes very strong at 5−10 μmol L^{-1} (Figure 5). In the presence of 5 μmol L^{-1} oligomycin, the flip acceleration by 0.05 mmol L^{-1} of extracellular Ca^{2+} (and ionophore) is comparable to that of 0.5 mmol L^{-1} Ca^{2+} in the absence of oligomycin (data not shown). These stimulated translocations in the presence of oligomycin and glibenclamide are suppressed by DTE or DIDS pretreatment (data not shown). Verapamil (50−200 μmol L^{-1}), which inhibits transport via both P-glycoprotein and MRP1 (*39*) but not that via ABCA1 (*38*), has no significant effect on the flop (data not shown).

Interestingly, clotrimazole (Figure 5), a potent inhibitor of the Ca^{2+}-activated K^+ channel (*41*, *42*), produces a concentration-dependent inhibition of the Ca^{2+}-activated translocation. Inhibition starts at 10 μmol L^{-1} and reaches about 85% at 100 μmol L^{-1} ($IC_{50} = 30$ μmol L^{-1}). At this high concentration, clotrimazole suppressed the oligomycin-stimulated Ca^{2+}-activated translocation (data not shown).

The effects of glibenclamide, oligomycin, and clotrimazole could also be demonstrated when the Ca^{2+}-activated flop was measured in the presence of MK571, which selectively inhibits translocation via MRP1 (*43*), instead of vanadate (data not shown). Inhibitor and stimulator properties comparable to those described for the Ca^{2+}-activated flop of NBD-PS could also be shown for the Ca^{2+}-activated flip of NBD-PC and the flip of NBD-PS in the presence of vanadate (data not shown). This substantiates the symmetry of the scrambling mechanism.

Membrane Phospholipid Asymmetry. To confirm that Ca^{2+}-activated flip-flop goes along with scrambling of membrane phospholipids, we looked for changes of their asymmetric distribution. To this end, cells were pretreated with Ca^{2+}/ionophore, flip rates were normalized by removal of Ca^{2+} and the ionophore from the cells (see Experimental Procedures), and the phospholipid composition of the outer membrane leaflet was analyzed by a treatment of cells with phospholipase A_2 (*1*−*3*, *34*). Although a gradual increase of cell lysis during exposure of cells to the phospholipase precluded saturating enzymatic cleavage, the advantage of this classic method is that it provides quantitative data on the fractional content of the three major membrane glycerophospholipids in the outer membrane leaflet. Exposure to 0.5 mmol L^{-1} Ca^{2+} increases the fractions of cleaved PE and PS (Table 2) from, respectively, 7 and 0% in control cells to 20 and 17% without significant changes in the fraction of cleaved PC (about 50%). These data correspond to previous results (*2*, *3*). The fractions of cleaved PE and PS further increase to, respectively, 36−40% and 28−34% following a pretreatment of the cells with NEM or PAO prior to Ca^{2+} loading, in line with the observed stimulation of the Ca^{2+}-activated flip and flop by these SH reagents (Table 1). A pretreatment of cells with DIDS before Ca^{2+} loading strongly reduces the fractions of cleaved PE and PS (Table 2), reflecting suppression of the Ca^{2+}-induced scrambling of membrane phospholipids. This is again in line with the observed DIDS effects on Ca^{2+}-activated flip and flop.

Echinocytosis. It has been known for many years that Ca^{2+} loading of erythrocytes transforms biconcave cells into echinocytes and finally leads to spheroechinocyte formation and exovesiculation (*44*−*47*). It seems reasonable to explain this shape transformations in terms of the bilayer couple hypothesis (*34*), i.e., that this change of shape is due to a Ca^{2+}-induced redistribution of membrane phospholipids and consecutive expansion of the outer leaflet of the bilayer with respect to the inner one. In this case, scramblase inhibition

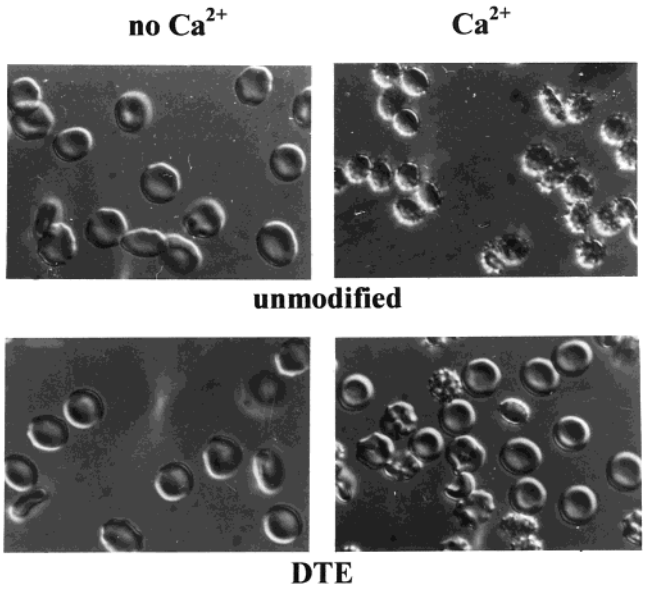

FIGURE 6: Suppression of Ca^{2+}-induced echinocytosis by inhibition of phospholipid scramblase activity. Control cells and cells treated with 10 mmol L^{-1} DTE (45 min, 37 °C) were incubated in KNHS either in the absence or in the presence of 0.5 mmol L^{-1} Ca^{2+} and 10 μmol L^{-1} A23187, and cell shape was examined by interference contrast microscopy.

should suppress echinocyte formation. DIDS could not be used for such experiments since it acts echinocytogenic itself (*48*). However, after inhibition of phospholipid scrambling by DTE which does not significantly affect the shape of normal biconcave cells (*49*), formation of echinocytes by Ca^{2+} loading was considerably reduced. Results of a typical experiment are shown in Figure 6. To minimize interference of the evaluation of shape transformations by microvesiculation, experiments were carried out in a K^+-rich medium which is known to suppress microvesiculation (*45*). In the absence of Ca^{2+}, untreated cells and DTE-pretreated cells are essentially biconcave, with mean morphological indices (MMI's) of, respectively, +0.19 and +0.12. After loading with Ca^{2+}, untreated cells are spheroechinocytes with a MMI of +3.6 whereas DTE-pretreated cells are largely biconcave with a MMI +0.7. This inhibition by DTE strongly suggests that membrane phospholipid scrambling is involved in Ca^{2+}-induced echinocytosis.

Microvesiculation. It should be kept in mind that measurements of Ca^{2+}-activated flip-flop were carried out routinely in a high K^+ medium (see Experimental Procedures). This medium is known to prevent cell shrinkage (*45*) resulting from loss of electrolytes and water upon activation of the Ca^{2+}-activated K^+ channel (*50*) and thereby to suppress the yield of microvesicles (*45*).

To study effects of scramblase inhibitors on Ca^{2+}-induced microvesiculation, cells were incubated in a medium containing Na^+ at high concentration and K^+ at low concentration. Under these conditions, Ca^{2+}-induced microvesiculation was found (Table 4) to be inhibited by DIDS and TNBS (data not shown) as well as DTE, and to be stimulated by NEM. Similar results were obtained when NBD-PS was used to label the inner membrane leaflet (see Experimental Procedures) instead of the use of NBD-SM to label the outer membrane leaflet (data not shown). The inhibition of microvesiculation by DIDS and TNBS could be due to their inhibitory effects on anion transport via AE1 which suppress the salt loss from the cells and thereby prevent cell shrinkage (*51*). Since DTE, on the other hand, does not prevent cell shrinkage, the observed inhibition of microvesiculation by this agent is probably due to the suppression of phospholipid scrambling. To confirm the important role of cell shrinkage in microvesiculation (*41*) of the modified cells, cell shrinkage due to salt loss was suppressed by an increase of the K^+ concentration in the medium. Indeed, in the high K^+ media, the yield of microvesicles proved to be highly reduced. Inhibition of scrambling by DTE further reduces microvesiculation (Table 4).

Table 3: Inhibition by DIDS and Stimulation by NEM or PAO of Ca^{2+}-Activated Scrambling of Endogenous Membrane Phospholipids[a]

pretreatment		exposure to	% cleavable by phospholipase			
first	second	mmol L^{-1} Ca^{2+}	PC	PE	PS	
–	–	–	53	6	0	(3)
–	–	0.5	53	20	17	(3)
–	DIDS	0.5	56	7	2	(3)
NEM	–	0.1	49	36	34	(4)
NEM	DIDS	0.1	51	18	11	(1)
NEM	–	0.2	49	36	34	(1)
NEM	–	0.5	50	6	32	(3)
NEM	DIDS	0.5	55	13	10	(2)
PAO	–	0.5	48	40	28	(2)
PAO	DIDS	0.5	47	24	16	(2)

[a] Erythrocytes were treated at 37 °C with NEM (0.8 mmol L^{-1}, 20 min) or PAO (0.2 mmol L^{-1}, 20 min) followed by DIDS (50 μmol L^{-1}, 30 min). Modified cells and corresponding control cells were incubated (30 min, 37 °C) with 0.5 mmol L^{-1} Ca^{2+} and 10 μmol L^{-1} A23187. After removal of intracellular Ca^{2+} and ionophore (see Experimental Procedures), the distribution of the various major glycerophospholipids between the membrane leaflets was determined by measuring their accessibility toward cleavage by bee venom phospholipase A_2 (see Experimental Procedures). Number of experiments is given in parentheses.

Table 4: Effects of Inhibition and Stimulation of Scramblase on Microvesicle Formation by an Increase of Intracellular Ca^{2+} [a]

	% of inserted NBD-SM in microvesicles	
modification	Na^+	K^+
none	15 ± 4	7 ± 2
DTE	7 ± 3	3 ± 1
DIDS	4 ± 2	3
NEM	24	5
NEM, then DTE	12	5

[a] Erythrocytes were pretreated at 37 °C with 50 μmol L^{-1} DIDS (30 min), 10 mmol L^{-1} DTE (45 min), 0.8 mmol L^{-1} NEM (20 min), or NEM followed by DTE. After insertion of NBD-SM into the outer membrane leaflet, controls and modified cells were resuspended in Na^+-rich (NHS) or K^+-rich (KNHS) medium containing 0.5 mmol L^{-1} Ca^{2+} and 10 μmol L^{-1} ionophore. The release of microvesicles from modified and control cells was then determined after 30 min of incubation (see Experimental Procedures). Data represent mean values from at least 3 experiments with the SD in the case of 4 or more experiments.

DISCUSSION

Effects of Inhibition and Stimulation on Ca^{2+}-Activated Inward Flip and Outward Flop of Phospholipids. In the present work, Ca^{2+}-activated phospholipid scrambling activity is shown to be suppressed (90%) by pretreatment of cells with the amino reagents DIDS or TNBS as well as the disulfide-reducing reagent DTE, which is also effective when present during flip-flop measurements. Reactions of DIDS

and TNBS are limited to extracellular domains of membrane proteins since DIDS is an impermeable amino reagent (*36*) and penetration of TNBS into erythrocytes is a slow process (*52*). Assuming the involvement of the human scramblase in Ca^{2+}-activated flip-flop, the reaction site could be lysine at position 314 of the exoplasmic nonapeptide chain of the scramblase (*20*). In line with this idea, the Ca^{2+}-activated flip in erythrocytes from mice, which are known to lack this exoplasmic chain (*21*), was only inhibited 39 ± 8% ($n = 3$; data not shown) after the DIDS treatment. Covalent modification of the scramblase by DIDS and TNBS may be postulated to prevent the Ca^{2+}-induced conformational change of the scramblase (*18*) which is required for translocase activity.

Interestingly, the inhibitory effects of DIDS and TNBS on scramblase activation are abolished by a transient increase of the intracellular Ca^{2+} concentration though the ability to activate the scramblase by Ca^{2+} is retained. This is evidence for an irreversible or only slowly reversible conformational change of the scramblase after Ca^{2+} binding. Such a conformational change may result in either an increase of the depth of insertion of the exofacial peptide chain into the membrane preventing reaction or an alteration of the mechanism of Ca^{2+}-dependent activation of translocation. Since inhibition of proteolytic degradation of membrane proteins by *N*-tosyl-L-phenylalanine chloromethyl ketone did not affect the Ca^{2+}-induced loss of the inhibitory effect of DIDS (data not shown), proteolysis is unlikely to be involved.

The strong suppression of scramblase activation following treatment of cells with the disulfide reducing agent DTE and reactivation by the SH oxidizing agent diamide (*53, 54*) is evidence for an essential role of intramolecular disulfide bonds within monomeric scramblase or intermolecular disulfide bonds between monomers for the activity of the scramblase.

Scramblase stimulation by covalent modification of SH groups is evident from the enhancement of Ca^{2+}-activated flip (Figure 1) and enhanced exposure of endogenous phosphatidylserine to the outer membrane leaflet (Table 3). This stimulation is in line with the reported enhancement of the Ca^{2+}-stimulated procoagulant state of erythrocytes by NEM (*55*). Similar stimulations are observed following treatment of cells with PAO, a reagent forming adducts with vicinal SH groups (*37*), and the SH oxidizing agent (*53*) diamide. The type of modification is therefore not very important for the stimulation. Interestingly, Ca^{2+}-activated lipid translocation in human platelets has been reported to be inhibited by a short (2−3 min) treatment with another SH reagent, pyridyldithioethylamine (*56*). It is concluded that several SH groups of the scramblase are present in the reduced form and are allosterically connected to the lipid translocation site.

In addition to the covalent modifiers, we also found noncovalent modifiers of scramblase activity. The suppression by clotrimazole, an inhibitor of Ca^{2+}-activated K^+ channel (*41, 42*), takes place in the same concentration range as that reported for ethanimidothioic acid (*57*), but both agents are structurally different. The inhibitory effect of clotrimazole on the scramblase could be reverted by washing of erythrocytes with medium (data not shown), whereas that of ethanimidothioc acid has been reported to be irreversible (*57*). The mechanism of stimulation of scramblase activity by oligomycin is likely to be different from its known inhibitory effects on the Na^+,K^+-ATPase (58), the Ca^{2+}-activated K^+ channel (*40*), and transport via various ABC proteins (*38, 39*). Oligomycin may be postulated to enhance Ca^{2+} binding to the EF hand of the scramblase or to stimulate the conformational change of the scramblase required for lipid translocation. Addition of oligomycin decreases the Ca^{2+} concentration required to obtain a given flip rate (data not shown). An enhanced susceptibility of the scramblase toward Ca^{2+} under physiological conditions might be one possible explanation for the high intracellular Ca^{2+} concentrations required to activate scrambling by Ca^{2+}/ionophore in erythrocytes.

The lack of inhibition of the Ca^{2+}-activated outward flop of NBD-PS by addition of glibenclamide and DIDS during flop measurement, which have been reported to inhibit transport via ABCA1 (*30, 38*), excludes a significant contribution of this transporter to Ca^{2+}-activated translocation in human erythrocytes. This conclusion is supported by the observed symmetry of effects of inhibitors and stimulators on inward and outward translocation.

Ca^{2+}-activated flip has been reported for structurally diverse exogenous lipid probes such as phospholipids, lysophospholipid (*3*), platelet activating factor (*59*), palmitoylcarnitine (*3*), and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (*7*) and is shown in the present work for NDP1 and NDP2. This demonstrates the low specificity of the scramblase. It might be speculated that the scramblase acts as a Ca^{2+}-dependent perturber of the lipid bilayer which produces unspecific flip sites for (phospho)lipids. However, structural defects are different from those postulated (*60*) to be produced by perturbation of the bilayer following insertion of amphiphiles and channel-forming agents into the lipid bilayer or covalent modification of membrane proteins as well as electroporation of the membrane barrier. In the latter cases, defect formation goes along with formation of leak pathways for polar solutes (*60*) which are not observed during an increase of intracellular Ca^{2+} (*61*).

Echinocytosis and Microvesiculation. The scramblase-mediated flip of sphingomyelin from the outer to the inner membrane leaflet is much slower than flips and flops of the glycerophospholipids (*5*, *35*). This should lead to an excess of outer leaflet surface area that has been suggested to be the cause of echinocytosis and microvesiculation (*5*). Alternatively, the breakdown of a pool of inner leaflet polyphosphoinositides by Ca^{2+}-activated phospholipase C has been discussed to be sufficient to produce the decrease of inner membrane surface area required for echinocytosis (*47*). The latter possibility seems to be unlikely for the following reasons. First, Ca^{2+}-induced echinocytosis is reverted upon removal of intracellular Ca^{2+} and incubation with nutrients whereas the polyphosphoinositide pool cannot be restored (*47*). Second, echinocyte formation can be repeated upon a renewed increase of intracellular Ca^{2+} (Stephan Schwarz, personal communication). On the other hand, the inhibition of Ca^{2+}-induced echinocytosis by scramblase inhibition with DTE demonstrated in Figure 6 supports a causal involvement of net shifts of phospholipids by scrambling in echinocytosis. Under the assumption that scrambling of inner leaflet phosphatidylethanolamine compensates for scrambling of outer leaflet phosphatidylcholine and that scrambling of outer leaflet sphingomyelin is considerably less than that of inner

leaflet phosphatidylserine (*5*, *35*), a net gain of outer leaflet phospholipid of about 4% can be estimated for cells incubated with 0.5 mmol L^{-1} Ca^{2+} and ionophore (Table 3). Since this gain goes along with an equivalent loss of inner leaflet phospholipid, this would result in a 8% excess of the phospholipid area of the outer leaflet with respect to the inner leaflet. For the formation of early spheroechinocytes, an area difference of only 2.7 ± 1.1% has been postulated (*62*) to be sufficient. It seems, therefore, more than likely that the area difference between the outer and inner leaflet is the major cause of echinocytosis following an increase of intracellular Ca^{2+}.

The problems of correlations between phospholipid scrambling and microvesiculation have already been addressed earlier (*4*, *31*, *63*−*65*). On one hand, phospholipid scrambling and microvesiculation were claimed to be independent processes (*64*). On the other hand, phospholipid scrambling has been proposed to be an essential step in microvesiculation but not the underlying cause (*65*). Our results support the first proposal and require apposition of the second proposal. The concomitant inhibition by DTE of both phospholipid scrambling and microvesiculation (Table 4) suggests a stimulatory, but not essential, role of phospholipid scrambling in microvesiculation. Our data (Table 4) and previous (*45*) data clearly demonstrate that cell shrinkage plays a major role in microvesiculation.

ACKNOWLEDGMENT

We thank Dr. E. Bremen for synthesis of the lipid probe NDP2, Mr. F. J. Kaiser for photographic work, Mrs. H. Heidtmann for providing murine blood, and Prof. B. Deuticke for his expert advice and critical reading of the manuscript.

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BI0107492