Cholesterol Redistribution within Human Platelet Plasma Membrane: Evidence for a Stimulus-Dependent Event[†]

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ABSTRACT: The fluorescent analog NBD-phosphatidylethanolamine and the analogs of cholesterol NBD-cholesterol and cholestatrienol were used to study the distribution of these lipids within the plasma membrane bilayer of human platelets. The probes were incorporated into platelets using phosphatidylcholine donor vesicles. The distribution of NBD lipid and of cholestatrienol in the platelet plasma membrane bilayer was followed by quenching with dithionite and TNBS, respectively. The $t_{1/2}$ of cholestatrienol incorporation into platelet membranes was 39 min, and approximately 65% of the probe was quenched by addition of TNBS. When platelets were exposed to collagen or to ADP, a portion of the probe became inaccessible to quenching. Within 2 min of stimulation by collagen (10 μg/mL), the percentage of cholestatrienol fluorescence quenched by TNBS decreased to 45%. The fluorescent probe was not found to be associated either with the intracellular membranes or in the extracellular media after collagen stimulation. Similar data were obtained with NBD-cholesterol, but the decrease in accessibility of this probe to quenching was considerably slower. The redistribution of endogenous membrane cholesterol was also measured using cholesterol oxidase. Exposure of platelets to collagen decreased the accessibility of endogenous membrane cholesterol to enzymatic oxidation with cholesterol oxidase. Taken together, the foregoing observations are consistent with the stimulus-dependent translocation of cholesterol out of the outer monolayer. Coincident with the redistribution of cholesterol is the reciprocal movement of NBD-phosphatidylethanolamine into the outer monolayer. In the presence of the chaotropic agents urea and guanidine HCl, the movement of cholestatrienol upon collagen stimulation was prevented, but the redistribution of NBD-phosphatidylethanolamine was still detected. We propose that cholesterol translocates to the inner platelet monolayer following collagen stimulation, but the possibility that the sterol moves laterally within the outer membrane monolayer cannot be rigorously excluded.

The physical properties of phospholipids and neutral lipids, such as cholesterol, within membrane monolayers, and how they influence each other are the major determinants of the dynamic properties of the membrane bilayer as a whole. Plasma membrane phospholipids are asymmetrically distributed across the membrane bilayer; generally, the outer monolayer is enriched in choline-containing lipids, phosphatidylcholine and sphingomyelin, while the amine-containing phospholipids, phosphatidylserine and phosphatidylethanolamine, are found predominately in the inner monolayer [for review, see Op den Kamp (1979), Loh and Huestis (1993), Rothman and Lenard (1977) Lenard and Rothman (1976), De Jong and Ott (1993)]. The establishment and maintenance of phospholipid asymmetry was first recognized to occur through a protein-mediated lipid transfer process [for review, see Devaux (1990)]. Since that time, translocase activity has been identified or implicated in platelets, erythrocytes (Sune et al., 1987; Daleke & Huestis, 1985), synaptosomes (Zachowski & Gaudry-Talarmain, 1990), chromaffin granules (Zachowski et al., 1989), and various other cell types (Wu & Hubbell, 1993; Muller, et al., 1994). Half-times for the transmembrane movement of phospholipids have been reported to be a few minutes in *Bacillus megaterium* (Rothman & Kennedy, 1977), 2−10 h in erythrocytes (Crain & Zilversmit, 1980), and several days in influenza virus (Rothman et al., 1976).

Cholesterol, a ubiquitous and essential component of mammalian cell membranes, is also asymmetrically distributed in a number of cells [for review, see Dawidowicz (1987) and Lange (1992)]. Work on the role of membrane cholesterol has focused primarily on the interactions between cholesterol and membrane phospholipids and proteins [for review, see Yeagle (1989)], the lateral or transmembrane distribution of this sterol [for review, see Schroeder (1985) and Schroeder et al. (1991a)], and the rates of cholesterol movement between and through cell membranes [for review, see Dawidowicz (1987), Lange (1992), and Schroeder and Nemecz (1990)]. Half-times of cholesterol movement have been reported from 3 s in erythrocytes (Lange et al., 1981) (under nonequilibrium conditions), 50−130 min in erythrocytes using [^{3}H]cholesterol vesicle exchange experiments (equilibrium conditions) (Brasaemle et al., 1988), and days in influenza virus (Lenard & Rothman, 1976). The large variations in cholesterol translocation rates may be due to the different techniques utilized (equilibrium versus nonequilibrium conditions) or may reflect the unique properties

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of the various membrane systems studied (Schroeder & Nemecz, 1990).

Phospholipid asymmetry can affect the distribution and exchange of cholesterol between membranes (House et al., 1989; Backer & Dawidowicz, 1981; Yeagle & Young, 1986). Model membrane studies suggest that membranes enriched with phosphatidylethanolamine provide a thermodynamically unfavorable environment for cholesterol. Thus, introduction of phosphatidylethanolamine into a membrane may cause the cholesterol to partition into a more favorable environment, for example, one rich in phosphatidylcholine (Yeagle & Young, 1986; Yeagle, 1987; Patzer et al., 1978). When cholesterol must coexist with phosphatidylethanolamine, the sterol may tend to form membrane domains of its own, with either cholesterol-rich or cholesterol-poor regions in a membrane (Gordon & Mobley, 1984; Gordon et al., 1983). Such cholesterol domains may be responsible for restricted phospholipid domains (Gordon et al., 1978, 1983; Curtain et al., 1978; Sauerheber et al., 1977; Verma & Wallach, 1975). Recent evidence suggests that preferential interactions between various phospholipid classes and cholesterol may define cholesterol pools in epithelial plasma membranes (El Yandouzi & LeGrimellec, 1992).

Human platelets offer a unique biological system in which to study the effects of a stimulus-dependent disruption of phospholipid asymmetry on membrane cholesterol asymmetry. Phospholipid asymmetry in platelet membranes is disrupted consequent to stimulation with collagen, thrombin, and ionomycin, all of which promote platelet aggregation. Upon stimulation, phosphatidylserine and phosphatidylethanolamine are postulated to translocate from the inner to the outer monolayer of the plasma membrane (Schick et al., 1976; Schroit & Zwaal, 1991; Bevers et al., 1982; Cha et al., 1977). This translocation is involved in the hemostatic process to mediate the formation of a prothrombinase complex (Bevers et al., 1982). Basse et al. (1993) have shown that half of the phosphatidylserine and phosphatidylethanolamine present in platelet membranes redistributes upon platelet activation. Recently, Gaffet et al. (1995) have shown that a directionally specific aminophospholipid outflux redistributes nearly half of the phosphatidylserine into the outer monolayer within 2 min of activation by A23187. Others, however, have suggested that phosphatidylcholine influx accompanies aminophospholipid outflux, resulting in a scrambling of the plasma membrane (Smeets et al., 1994; Williamson et al., 1995). This lipid scrambling may be mediated by a scramblase (Zwaal et al., 1993; Galli & Bevers, 1994) or may be due to vesicle shedding during strong activation (Comfurius et al., 1990; Schroit & Zwaal, 1991).

In this report we investigated the effect of platelet stimulation on the distribution of cholesterol in platelet plasma membrane. We have used two fluorescent sterols [cholesta-5,7,9(11)-triene 3β ol (cholestatrienol) and 7-nitrobenz-2-oxa-1,3-diazol-4-yl-cholesterol (NBD-cholesterol)] and susceptibility to cholesterol oxidase to measure cholesterol distribution within membrane monolayers. These three techniques all support a stimulus-dependent redistribution of cholesterol in human platelet plasma membrane. We show further that this translocation can be modulated by treatment of platelets with chaotropic agents and with agents that deplete cell ATP.

EXPERIMENTAL PROCEDURES

Platelet Isolation and Additional Assays. Platelets were isolated from platelet-rich plasma obtained from the American Red Cross. The red blood cells are removed from the platelet rich plasma by centrifugation at 750 rpm and the platelets collected by centrifugation at 2500 rpm for 20 min. The platelets are resuspended in NaCl (145 mM), KCl (5 mM), $MgSO_4$ (1 mM), and HEPES (10 mM), at pH 7.4 (platelet buffer) and filtered on a column of 40 mL of Sepharose 4B (Pharmacia) to remove remaining plasma (Carvalho et al., 1974). The eluted platelets are centrifuged at 750 rpm to remove any residual erythrocytes, suspended in platelet buffer, and assayed for cholesterol (Allain et al., 1974), phospholipid phosphate (Bartlett, 1959), and total protein (Lowry et al., 1951). Platelet cholesterol concentrations averaged 0.398 $\pm$ 0.037 mol of cholesterol/mol of phospholipid P_i. Platelet ATP content was assayed using the luciferin-luciferase method (Sigma Chemical Co., St. Louis, MO) on supernatants of platelet extracts prepared as described by Daleke and Huestis (1985).

Incorporation of Fluorescent Probes. Head group labeled phosphatidylethanolamine, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-PE, Molecular Probes, Eugene, OR), was incorporated using large phosphatidylcholine−cholesterol−NBD-PE unilamellar donor vesicles and a dilute platelet suspension to prevent aggregation, essentially as described by Williamson et al. (1992). NBD-cholesterol (Molecular Probes, Eugene, OR) and cholestatrienol (generously supplied by Dr. Philip Yeagle) were incorporated into platelet membranes using small unilamellar vesicles prepared from phosphatidylcholine (egg) (Avanti Polar Lipids), cholesterol, and NBD-cholesterol or cholestatrienol. The lipids were dissolved in acetonitrile, dried under nitrogen, lyophilized to remove trace amounts of solvent, dispersed in buffer, and sonicated to clarity. Large and multilamellar phospholipid vesicles were removed by centrifugation at 45 000 rpm for 30 min (Barenholtz et al., 1977). The cholesterol concentration of the exchange vesicles was 0.40 mol of cholesterol/mol of phospholipid, a value selected so as not to enrich or deplete the platelet membranes of endogenous cholesterol. NBD-cholesterol or cholestatrienol exchange vesicles were incubated with platelets for up to 3 h at 37 °C (House et al., 1989). Following incubation, the platelets were recovered by centrifugation at 2500 rpm for 15 min, resuspended in platelet buffer, and centrifuged a second time.

In these studies the phospholipid exchange vesicles routinely contained 2−4 mol % cholestatrienol relative to vesicle phospholipid. Upon incorporation, the ratio of cholestatrienol was determined to be 0.2−0.5 mol % relative to platelet phospholipid phosphate. If all of the observed cholestatrienol fluorescence associated with the platelets was due to vesicle adsorption, then at least 10% of the exchange vesicles would be adsorbed onto the surface of the platelet.

[1] Abbreviations: NBD-cholesterol, (7-nitrobenz-2-oxa-1,3-diazol-4-yl)cholesterol; cholestatrienol, cholesta-5,7,9(11)-triene-3β-ol; ABD, 7-amino-2,1,3-benzoxadiazol-4-yl; ADP, adenosine diphosphate; TNBS, 2,4,6-trinitrobenzenesulfonic acid; PE, phosphatidylethanolamine; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt; SUVs, small unilammellar vesicles; P_i, phosphate; cholest-4-en-3-one, cholestenone.

This degree of vesicle adsorption would have increased the platelet phospholipid to protein ratio by 5−10%, and this was not observed. Additional evidence arguing against vesicle sticking to platelets comes from experiments using donor vesicles containing 2−4 mol % *N*-(lissamine Rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (N-Rh-PE), a nonexchangeable fluorescent lipid, as described by Sleight and Abanto (1989). The amount of N-Rh-PE associated with the cells and the ratio of fluorescence between cholestatrienol and N-Rh-PE was measured. The maximum amount of cholestatrienol fluorescence associated with the cells due to vesicle adsorption was calculated (Sleight & Pagano, 1984) to be between 0.5% and 1% after the wash steps described. NBD-cholesterol was incorporated at probe levels such that its final intramembrane concentration did not exceed 0.2−0.50 mol % relative to phospholipid. The platelet membrane phospholipid concentration in the fluorescence experiments was 0.36 ± 0.04 μmol of P_i/mL of cells. NBD-cholesterol was incorporated into BALB 3T3 murine fibroblasts using the vesicle exchange technique as described for platelets.

Fluorescence Measurements. All fluorescence measurements were performed on a Perkin Elmer LS50B spectrofluorometer. Prior to the start of each experiment, $CaCl_2$ was added to the platelet suspension to a final concentration of 1.0 mM, and the platelets were transferred to cuvettes placed in a water jacketed turret at 37 °C. Fluorescence of NBD lipids was measured with excitation at 470 nm and emission at 525 nm. NBD-PE and NBD-cholesterol distribution was determined using a dithionite quenching assay as described (McIntyre & Sleight, 1991). The initial fluorescence (designated as F_o) was recorded; dithionite was then added and the fluorescence decreased to a new value as the NBD probe present in the outer monolayer was quenched. The fluorescent value F_r was recorded after the reaction was complete, and the fluorescence remained unchanged for 10 s. The addition of Triton X-100 caused a further decrease in fluorescence, such that essentially all of the remaining fluorescence was destroyed. When Triton X-100 was added prior to dithionite treatment, the addition of dithionite resulted in the quenching of 100% of the NBD fluorescence. Both fluorescence intensities (F_r and F_o) were corrected for the background fluorescence of unlabeled platelets (designated F_{app}), which did not change during the course of the experiments. The amount of NBD-lipid accessible to the dithionite was calculated from the decrease in cell-associated fluorescence after quenching with dithionite using the following formula (McIntyre & Sleight, 1991).

$$\% \text{ accessible} = \{1 - [(F_r - F_{app})/(F_o - F_{app})]\} \times 100$$

Cholestatrienol fluorescence was measured at 390 nm after excitation at 324 nm. Fluorescence was quenched with 2,4,6-trinitrobenzenesulfonic acid (TNBS) by energy transfer and confirmed as a shift in fluorescence intensity (Schroeder et al., 1979). The quenching reaction was followed for 120 s. When TNBS quenching was conducted at 4 °C (nonpenetrating conditions; Schroeder, 1981), the percentage of fluorescence quenched was identical to that observed at 37 °C, suggesting that negligible amounts of TNBS had penetrated the platelets and quenched probe fluorescence localized to the inner monolayer at either temperature. Quenchable cholestatrienol was calculated using the equation given above for dithionite quenching of NBD lipids, again correcting for background fluorescence of unlabeled platelets (McIntyre & Sleight, 1991). Platelet aggregation was not detected during these incubations.

Platelet Lysis and Recovery of Plasma and Intracellular Membranes. Platelet plasma membranes were separated from intracellular membrane fractions following hypotonic lysis of platelets loaded with cholestatrienol as described (Barber & Jamison, 1971; Harmon et al., 1992). Cholestatrienol fluorescence associated with each fraction was measured before and after TNBS quenching.

Cholesterol Oxidase Treatment. Platelets were incubated with cholesterol oxidase (*Nocardia sp.*, Calbiochem, La Jolla, CA) (5 units/mL platelets) for varying periods of time as indicated in the table or figure legends. In the experiment shown in Table 3, platelets were exposed to collagen for 5 or 10 min before introduction of cholesterol oxidase into the incubation; in other experiments, platelets were treated with collagen and cholesterol oxidase simultaneously. The incubation was terminated with the addition of methanol and the lipids were extracted into chloroform (Folch et al., 1957). The chloroform was evaporated to dryness under a stream of nitrogen, and the lipids were redissolved in hexane and analyzed by HPLC on a C-18 reverse-phase column eluted with methanol at a flow rate of 1.0 mL/min. Cholestenone was detected as absorbance at 240 nm and quantified following peak integration by comparison with known amounts of authentic standards (Sigma, St. Louis, MO). All extracts contained comparable amounts of lipid based upon assay of phospholipid phosphate. In the cholesterol oxidase experiments presented, the average phosphate concentration of the cells was 0.89 ± 0.06 μmol of phospholipid P_i/mL of cells.

Kinetics of Cholestatrienol Incorporation into Platelet Plasma Membrane. The exchange of cholestatrienol into platelet membranes from SUVs to platelet membranes was followed over time at 37 °C. At various times, an aliquot was removed from the incubation and diluted with platelet buffer, and the platelets were separated from the vesicles by centrifugation. The fluorescence associated with the platelets was measured as described above. As seen in Figure 1, the fluorescent sterol was readily incorporated into platelets. The temporal relationship of cholestatrienol incorporation was analyzed using Sigma Stat (Jandel Scientific). Both a multilinear regression and a polynomial regression analysis yielded similar statistical results. The cholestatrienol incorporation data best fit a single-exponential curve indicative of a first order relationship, with a $p < 0.00001$, a coefficient of determination equal to 0.959, and an F value of 189. The suitability of a second or third order fit between the cholestatrienol concentrations and time was also analyzed in detail, and, in these instances, the p values were 0.51 and 0.80, respectively. The incorporation followed a first order kinetic process, and the $t_{1/2}$ of this process was determined to be 39 min from a plot of ln $(F_{(t)}/F_0)$ versus time (not shown). The slope of the line represents $(-)k$ and the $t_{1/2}$ is calculated from the mathematical relation $t_{1/2} = 0.693/k$. The time course for the amount of cholestatrienol that is accessible and inaccessible to TNBS quenching is shown in (Figure 2). After 45 min of incubation, there is no further change in the amount of fluorescence associated with the TNBS quenchable cholestatrienol. There is, however, an increase in the fluorescence associated with the inaccessible

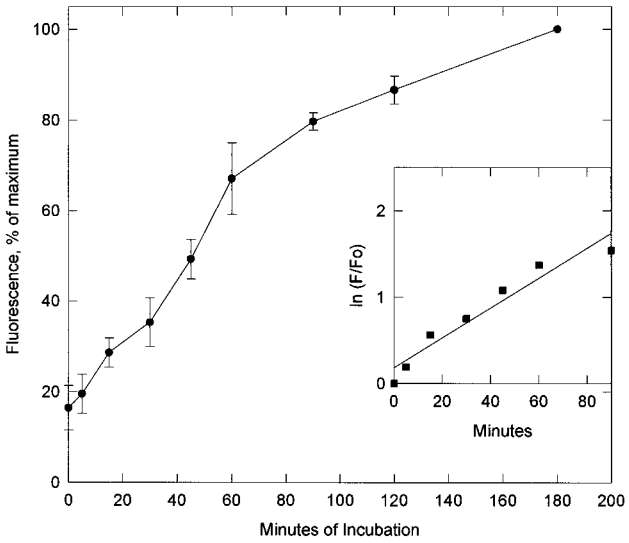

FIGURE 1: Uptake of cholestatrienol into platelets. Cholestatrienol incorporation was measured exactly as described in Experimental Procedures. Each fluorescence value was corrected for the intrinsic fluorescence associated with a platelet suspension not loaded with cholestatrienol. The data are expressed as percentages of the maximum fluorescence value obtained after 180 min of incubation. The insert shows the ln of the ratio of each fluorescence value and the zero time fluorescence at the indicated incubation times. The slope of the calculated line is used to derive a half time of cholestatrienol loading of 39 min; $-k$ was calculated to be 0.0175 fluorescence units/min. Each value shown is the mean and standard error of three experiments, each performed on a different platelet preparation.

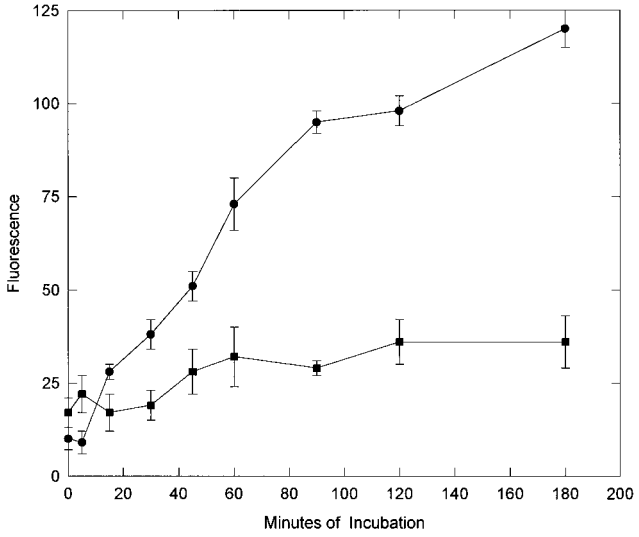

FIGURE 2: Translocation of cholestatrienol to the inner monolayer of platelets. Cholestatrienol incorporation was measured exactly as described in Experimental Procedures. The incubation was terminated by collecting the platelets by centrifugation. The platelets were resuspended in platelet buffer and cholestatrienol fluorescence measured before and after addition of TNBS. Each fluorescence value was corrected for the intrinsic fluorescence associated with a platelet suspension not loaded with cholestatrienol. Cholestatrienol that was accessible to TNBS quenching is given by the filled squares (■). Cholestatrienol that was inacessible (●) to TNBS quenching was calculated as the difference between total cell fluorescence and the value for TNBS quenchable cholestatrienol. Each value shown is the mean and standard error of three experiments, each performed on a different platelet preparation.

pool. These results suggest that, after approximately 45 min of incubation, the cholestatrienol is incorporating into a region of the platelet plasma membrane that is not accessible

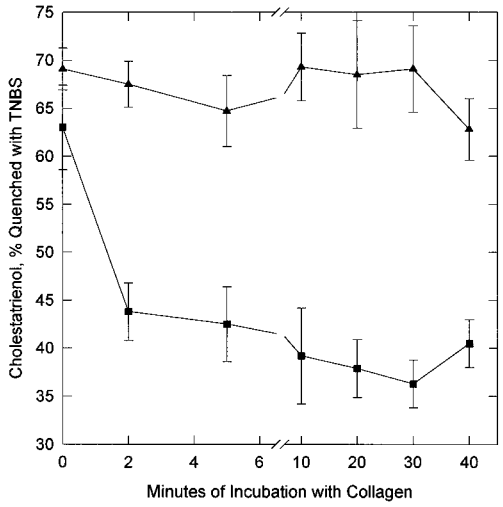

FIGURE 3: Collagen decreases the accessibility of cholestatrienol to quenching with TNBS. Platelets were loaded with cholestatrienol using SUVs as described and incubated in the absence (▲) or presence (■) of collagen (10 μg/mL) for the times indicated on the abscissa. The amount of cholestatrienol in the outer leaflet is determined as the decrease in cell-associated fluorescence after quenching with TNBS and calculated as described. Each value presented is the mean and standard error of four replicate experiments for platelets incubated in the absance of collagen and six experiments for platelets incubated with collagen. Each experiment was performed on a different platelet preparation.

to quenching, most likely the inner monolayer. All subsequent studies (unless otherwise noted) employed platelets that were incubated with cholestatrienol or NBD-cholesterol donor vesicles for 3 h, a time period that is at least 4 times the $t_{1/2}$ determined. Thus, incubations of this duration should ensure that equilibria between cholestatrienol in the donor and acceptor membranes, and between the outer and inner membrane monolayer, were established. Therefore, upon platelet activation, the stimulus-dependent redistribution of these probes could be followed. The kinetic parameters were determined using Sigma-Plot (Jandel Scientific). From these kinetic parameters, it is predicted that less than 0.5% of the probe would redistribute across the bilayer during quenching with TNBS during the short time course used in these studies.

RESULTS

Collagen Promotes an Apparent Movement of Fluorescent Cholesterol Probes. This study employed fluorescent sterol analogs to probe the asymmetric distribution of cholesterol in platelet plasma membranes following stimulation with collagen. The studies shown in Figure 3 used cholestatrienol, which is structurally similar to cholesterol and more importantly mimics cholesterol physiologically in cell membranes (Schroeder & Nemecz, 1990; Yeagle, 1989; Yeagle et al., 1990). In unstimulated cholestatrienol labeled platelets, between 65% and 68% of the probe's fluorescence was quenched with the addition of TNBS. The incubation of cholestatrienol labeled platelets at 37 °C in the absence of stimulation did not alter the percentage of fluorescent probe that was accessible to the TNBS (Figure 3). In contrast, exposure to collagen for only 2 min decreased the percentage of cholestatrienol accessible to TNBS to less than 45%. The percentage of the probe that could be quenched with TNBS changed very little over the remaining 38 min of incubation. Similar experiments were conducted using NBD-cholesterol labeled platelets. In these studies, 70% ± 8% of the probe

Table 1: Distribution of Cholestatrienol between Plasma Membranes and Intracellular Membranes Of Human Platelets[a]

	plasma membranes		intracellular membranes	
	control	collagen	control	collagen
fluorescence	154 ± 17.0	173 ± 39.0	13 ± 6.2	19 ± 9.9
% total	92.2 ± 4.3	90.1 ± 6.1	7.8 ± 3.3	9.9 ± 3.9
% TNBS accessible	38.4 ± 3.5	25.4 ± 3.9	ND	ND

[a] Cholestatrienol was incorporated into human platelets during incubation with freshly prepared SUVs for 3 h. The platelets were harvested, incubated with 10 μg/mL collagen for 10 min, and lysed in hypoosmotic buffer. The plasma membranes and the intracellular membranes were isolated and suspended at concentrations of 0.45 mM P_i and 0.14 mM phospholipid P_i, respectively. The fluorescences associated with each fraction were measured before and after addition of TNBS and corrected for the nonprobe fluorescence associated with each membrane fraction. Each value is the mean ± standard error of three separate experiments. ND, not determined.

Table 2: TNBS Quenching of Cholestatrienol in BSA Treated Platelets

	F_o	F_r	F_{app}	% outer monolayer
control	181 ± 21	140 ± 19	116 ± 17	63.1 ± 7.1
BSA treated	211 ± 9.8	205 ± 9.0	123 ± 8.4	6.8 ± 0.5

[a] Cholestatrienol was incorporated into human platelets during incubation with freshly prepared SUVs for 3 h. The platelets were recovered by centrifugation and treated with 2% defatted bovine serum albumin (BSA) for 5 min. The platelets were separated from the BSA-containing media by centrifugation and fluorescence measured before (F_o) and after treatment with TNBS (F_r). The fluorescence of unloaded platelets at the same cell concentration was recorded (F_{app}). Each value presented is the mean and standard error of three separate experiments.

was quenched upon addition of sodium dithionite in unstimulated platelets; only 41% ± 6% of the probe was quenched with sodium dithionite 40 min after exposure to collagen (10 μg/mL). Thus, exposure of platelets to collagen decreased the accessibility of the probes cholestatrienol or NBD-cholesterol to the quenching agents TNBS or sodium dithionite, respectively. NBD-cholesterol labeled fibroblasts (BALB 3t3 fibroblasts) stimulated with bradykinin (1.0 μM) showed no change in the accessibility of NBD cholesterol to dithionite, suggesting that the phenomenon observed in collagen stimulated platelets may not occur in all other cells.

A movement of fluorescent probe into the intracellular membranes consequent to collagen stimulation could also decrease the accessibility of the fluorescent sterol probes to the various quenchers. To test this possibility, the distribution of cholestatrienol in plasma membrane and intracellular fractions was determined following platelet lysis and sucrose density gradient centrifugation (Barber & Jamieson, 1971; Harmon et al., 1992). When the fluorescence associated with the plasma membrane and intracellular membrane fractions was compared (Table 1), 92.2% of the total quenchable cholestatrienol was recovered from the plasma membrane fraction (Barber & Jamieson, 1971). Virtually the same percentage (90.1%) of the cholestatrienol was recovered from the plasma membrane fraction of collagen stimulated platelets. Less than 10% of the fluorescence was associated with intracellular membranes, and, most importantly, this percentage was not affected by collagen treatment prior to lysis.

Interpretation of the foregoing experiments assumes that the quenching of probe fluorescence with dithionite or with TNBS does not itself cause a redistribution of the probe within the membrane and that only the fluorescence of probe confined to the outer monolayer is affected by the quencher. To test these assumptions, cholestatrienol was removed from the outer membrane monolayer of platelets by treatment with BSA. The fluorescence of cholestatrienol-loaded platelets was partially quenched with addition of TNBS, and the percent of the probe accessible to quenching was calculated to be 63.1% (Table 2). Following treatment with BSA, however, the fluorescence was quenched only slightly by addition of TNBS. The finding that TNBS decreased the fluorescence only slightly in BSA-treated platelets suggests the presence of TNBS did not cause the migration of inner monolayer cholestatrienol to the outer monolayer where it would be quenched. It also follows that the TNBS did not penetrate the platelets and quench inner monolayer fluorescence. The cholestatrienol fluorescence associated with BSA-treated platelets is slightly greater than in control platelets, and this difference probably reflects the higher cell concentration in the presence of BSA (control, 0.26 mM phospholipid P_i; BSA, 0.38 mM phospholipid P_i).

Collagen Decreases the Susceptibility of Cholesterol to Cholesterol Oxidase. The experiments presented above using the fluorescent sterol probes, cholestatrienol, and NBD-cholesterol point to a redistribution of a portion of the probe molecules within the membrane upon collagen stimulation of platelets. While the use of fluorescent sterol probes to study membrane cholesterol distribution and domains has been well documented [for review, see Schroeder et al. (1991a)], a caveat in the present studies is that neither experiment directly studied the distribution of endogenous cholesterol. To investigate the effects of collagen on the distribution of membrane cholesterol, the susceptibility of cholesterol to oxidation by cholesterol oxidase in stimulated and unstimulated platelets was determined.

The selective oxidation of outer monolayer cholesterol by cholesterol oxidase has been extensively documented by other investigators [for review, see Lange (1992), Dawidowicz (1987), Brasaelme et al. (1988), Brasaelme and Attie (1990), and Patzer et al. (1978)]. Upon exposure of platelets to cholesterol oxidase for 10 min, a portion of the total cell cholesterol, representing 3−10% of the total membrane cholesterol, was converted to cholest-4-en-3-one (cholestenone). This amount of cholesterol oxidase accessible cholesterol in platelets is consistent with work by Brasaelme and Attie (1990) on erythrocytes in which 3% of the total membrane cholesterol was converted by cholesterol oxidase in unmodified cells. When platelets were exposed to collagen for 5 or 10 min prior to addition of cholesterol oxidase, the formation of cholestenone was significantly decreased, suggesting that a portion of outer monolayer cholesterol had become inaccessible to cholesterol oxidase (Table 3). ADP (10 and 50 μM) also decreased the formation of cholestenone, but the magnitude of the ADP effect was considerably smaller than that of collagen (data not shown).

The cholestatrienol data presented in Figure 3 suggest that cholestatrienol redistributes within 2 min of incubation with collagen, and this result is confirmed by the data in Table 3. However, the incubation conditions used to treat cells with cholesterol oxidase in Table 3 gave an apparent maximum amount of cholestenone formed in the presence of collagen

Table 3: Cholestenone Formation in Untreated and Collagen Stimulated Platelets

incubation with collagen (min)	cholestenone formed (nmol/mL platelets)		
	control	collagen (10 μg/mL)	Δ
15	6.4 ± 1.0	3.8 ± 0.7	2.6 ± 0.6[b]
20	7.0 ± 0.7	4.7 ± 0.6	2.3 ± 0.3[b]

[a] Platelets were incubated at 37 °C in the absence or presence of collagen for a total of 15 or 20 min as indicated. Cholesterol oxidase (5 units/mL) was then added for the final 10 min of incubation. The reaction was terminated by addition of methanol and the lipids extracted into chloroform. Cholestenone content was quantified by absorbance at 240 nm following HPLC separation. Each value is the mean and standard error of 12 observations. [b] Mean difference is significantly greater than zero by paired Student's *t*-test, $p < 0.05$.

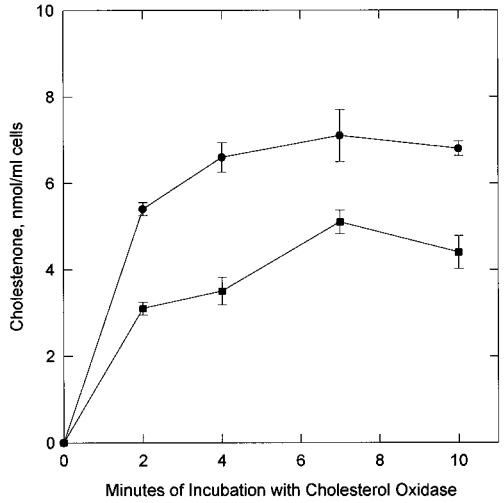

FIGURE 4: Collagen decreases the enzymatic conversion of endogenous cholesterol to cholestenone. Platelets were incubated in the absence (●) of presence (■) of collagen (10 μg/mL) and cholesterol oxidase (5 U/ml platelets) for the times indicated. The reaction was terminated by addition of methanol and the lipids extracted into chloroform. Cholestenone content was quantified by absorbance at 240 nm following HPLC separation. Each value presented is the mean and standard error of eight observations.

within 5 min. These two observations suggest that endogenous cholesterol may translocate on the same time scale (<5 min) as cholestatrienol. In the following set of experiments, collagen was present simultaneously with cholesterol oxidase for the time periods indicated. As seen in Figure 4, in the presence of collagen, there was 50% less cholestenone formed when compared with control conditions. Within 60 min, however, the amount of cholestenone formed in control and collagen-treated platelets was identical (8.06 ± 0.9 nmol/mL of cells in control and 8.97 ± 1.1 nmol/mL in collagen-stimulated cells). Thus it is anticipated that while collagen is able to render a portion of the platelet membrane cholesterol inaccessible to cholesterol oxidase, continued treatment with oxidase results in a redistribution of the remaining cholesterol such that the accessible pool in both collagen-treated and control platelets is similarly hydrolyzed. The possibility that collagen is acting through a nonspecific interaction between the hydrophobic regions of the collagen molecule and cholesterol oxidase was excluded by the observation that collagen did not alter the formation of cholestenone in phosphatidylcholine/cholesterol vesicles (30 mol% cholesterol) treated with cholesterol oxidase.

Effect of Iodoacetamide on Cholestenone Formation. Our next experiments sought to investigate the possible role of ATP in the collagen-induced redistribution of cholesterol. In these experiments platelets were incubated with iodoacetamide so as to lower the concentration of ATP in the cells (Sune et al., 1987). As seen in Table 4, in iodoacetamide-treated platelets, collagen (10 μg/mL) did not alter the amount of cholesterol that was accessible to cholesterol oxidase. Direct measurement of platelet ATP showed the content of ATP to be reduced by approximately 50% following treatment with iodoacetamide (control, 3.74 nmol of ATP/mL of cells; iodoacetamide, 1.98 nmol of ATP/mL of cells). These results suggest that, in the presence of iodoacetamide, collagen stimulation did not result in a redistribution of cholesterol from the outer monolayer.

Table 4: Cholestenone Formation in Iodoacetamide-Treated Platelets[a]

	cholestenone formed (nmol/mL platelets)		
	basal	collagen, 10 μg/mL	Δ
control	12.72 ± 1.91	9.23 ± 2.07	3.49 ± 1.32[b]
6 mM iodoacetamide	15.52 ± 2.51	14.81 ± 1.93	0.71 ± 0.77

[a] Platelets were incubated in the absence or presence of 6.0 mM iodoacetamide for 60 min. Collagen was then added to a final concentration of 10 μg/mL and the incubation continued for five min. Cholesterol oxidase (5 unit/mL) was added, and the platelets were incubated for another 10 min. The incubation was terminated by addition of methanol, and the lipids were extracted into chloroform. Cholestenone content was quantified by absorbance at 240 nm following HPLC. Each value presented is the mean and standard error of four replicate experiments. Each experiment was performed on a different platelet preparation and all determinations were in triplicate. [b] Mean difference is significantly greater than zero by paired Student's *t*-test, $p < 0.05$.

Redistribution of NBD-Phosphatidylethanolamine. To determine the correlation between cholesterol movement and platelet activation as defined by aminophospholipid translocation, we investigated the effect of collagen stimulation on NBD-PE redistribution in the platelet plasma membranes. In these studies, platelets were loaded with NBD-PE such that approximately 5% of the probe was located in the outer membrane monolayer as measured by dithionite quenching, a level similar to the amount of NBD-PS reported to be incorporated into platelets (Tilly et al., 1990) and slightly less than was incorporated into erythrocytes (Conner et al., 1990). Both our results and those of Tilly et al. (1990) are consistent with the presence of an amino phospholipid translocase in the platelets as shown (Sune et al., 1987).

When NBD-PE labeled platelets were stimulated with collagen (10 μg/mL, at 37 °C), the percent of NBD-PE that was quenched by dithionite increased dramatically (Figure 5). After 5 min of incubation, the percent of NBD-PE that was quenched increased by 50%, and by 2-fold after 30 min. This time course is similar to the time frame observed for the redistribution of cholestatrienol and endogenous cholesterol within platelet membranes. The time course of NBD-PE redistribution is similar to that seen for NBD-PS during platelet activation (Gaffet et al., 1995) This effect of collagen to increase the percent of platelet NBD-PE quenched with dithionite persisted after enzymatic hydrolysis of bound collagen with collagenase.

Collagen stimulation of NBD-PE labeled platelets treated with iodoacetamide showed no stimulus-dependent change in the amount of NBD-PE associated with the outer monolayer. In the presence of iodoacetamide, 12.66% ± 1.44%

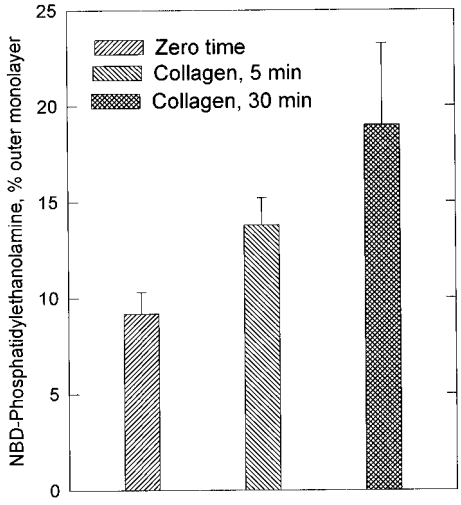

FIGURE 5: Collagen-induced redistribution of NBD-PE in platelet membranes. NBD-phosphatidylethanolamine was incorporated into platelets by incubation with the appropriate vesicles for 20 min as described in Experimental Procedures. NBD-PE labeled platelets were then incubated with collagen (10 μg/mL) at 37 °C for the times indicated. Each bar represents the mean and standard error of at least six independent observations.

of the NBD-PE was associated with the outer monolayer. Upon collagen stimulation, virtually the same percentage of NBD-PE (12.0% ± 1.06%) was determined to be in the outer monolayer.

Effect of Chaotropic Agents on Cholesterol Distribution. The correlation between NBD-PE translocation into the outer platelet monolayer and the translocation of cholesterol out of the outer monolayer calls into question the nature of the forces involved in the maintenance of cholesterol asymmetry and conversely the forces involved in the disruption of that asymmetry. The introduction of phosphatidylethanolamine into membranes creates a thermodynamically unfavorable environment for cholesterol. The distinction between phosphatidylethanolamine- and phosphatidylcholine-rich environments disappears in the presence of chaotropic agents (Yeagle & Young, 1986). When platelets were incubated with the chaotropic agents guanidine HCl or urea prior to treatment with collagen, the redistribution of NBD-cholesterol or cholestatrienol was no longer observed (Table 5). The stimulus-dependent translocation of NBD-PE, however, was not effected by the chaotropes, indicating that the inhibition of collagen-induced cholesterol translocation by urea and guanidine HCl cannot be attributed to the prevention of phosphatidylethanolamine translocation. Neither guanidine HCl nor urea altered the distribution of NBD-cholesterol or cholestatrienol in unstimulated platelets. The possibility that urea acts to denature collagen was addressed using ADP to mediate cholestatrienol translocation. Upon ADP stimulation, the amount of outer monolayer cholestatrienol decreased from 68.2% ± 6.5% (control) to 54.3% ± 4.8% in stimulated platelets. In urea-treated platelet, the amount of outer monolayer cholestatrienol was 71.6% ± 5.1% (control) and 74.0% ± 5.5% in ADP-stimulated cells. Thus both collagen- and ADP-dependent cholestatrienol translocation was prevented in the presence of chaotropic agents.

Table 5: Effect of Urea and Guanidine HCl on NBD-PE and Cholesterol Distribution upon Collagen Stimulation

	control	urea	guanidine HCl
cholestatrienol			
basal	68.3 ± 6.6	71.8 ± 5.1	74.0 ± 6.7
collagen	50.2 ± 4.6[b]	75.0 ± 5.5	74.3 ± 6.4
NBD-PE			
basal	6.7 ± 1.6	7.6 ± 1.3	6.9 ± 1.9
collagen	12.1 ± 1.9[b]	12.6 ± 1.6	11.9 ± 1.9
NBD-cholesterol			
basal	75.9 ± 3.2	81.4 ± 7.6	76.5
collagen	60.5 ± 6.3[b]	76.5 ± 4.8	75.5

[a] Platelets were isolated and labeled with either NBD-cholesterol, cholestatrienol, or NBD-PE as described. The labeled platelets were incubated at 37 °C for 5 min with 1.0 mM $CaCl_2$ and 10 μg/mL collagen in the presence or absence of urea or guanidine HCl, present at a final concentration of 1.0 M. Urea and guanidine HCl were added in five aliquots of 200 mM per aliquot in 1 min. The cholestatrienol data are the average and standard error of four separate experiments. The NBD-PE data are the average and standard errors of three separate experiments. The NBD cholesterol data are an average of two separate experiments in the case of guanidine HCL treatments and three separate experiments in control and urea-treated samples. [b] Significantly different from basal by paired Student's *t*-test, $p < 0.05$

DISCUSSION

The salient finding reported in this paper is an intramembrane redistribution of cholesterol upon stimulation of human platelets with collagen. This heretofore unreported phenomenon is based upon results obtained using two independent techniques. We have found that the accessibility of the fluorescent sterol probes cholestatrienol and NBD-cholesterol to quenching by TNBS and sodium dithionite, respectively, decreased by approximately 50% after stimulation of platelets with collagen. We also found that the susceptibility of platelet cholesterol to oxidation with cholesterol oxidase decreased by as much as 45% (Figure 4) upon collagen stimulation. When viewed collectively, the results from these three types of experiment suggest a redistribution of a fraction of platelet membrane cholesterol after stimulation with collagen. The results of these experiments are essentially the same irrespective of whether fluorescent probe or endogenous cholesterol redistribution was measured.

Our observation that collagen (or ADP) stimulation of human platelets causes a fraction of the endogenous cholesterol or fluorescent cholesterol probes to become inaccessible to oxidation by cholesterol oxidase or to quenching, respectively, suggests the existence of accessible and inaccessible pools of cholesterol in the membrane. The precise location of these pools is not known, but the simplest interpretation consistent with the present data is that cholesterol present in the inner monolayer is inaccessible; cholesterol in the outer monolayer is accessible. The presence of such pools is consistent with calorimetry studies following lipid phase separations which suggested the existence of cholesterol-rich and cholestero-poor membrane domains in these cells (Gordon et al., 1983). The experiments described herein do not support rigorous correlation between accessibility of probes to quenching agents and heterogenous cholesterol domains within platelet membranes. Additional experimentation employing more sophisticated fluorescence techniques is needed to investigate the existence of such membrane domains.

Analysis of the kinetics of cholestatrienol incorporation into human platelets yielded additional information about the distribution of cholesterol in the membrane. Cholestatrienol uptake was found to follow first order kinetics with a half-time of 39 min. Approximately 50% of the incorporated

cholestatrienol was associated with the inner monolayer within 45−60 min. Cholestatrienol, due to its "puckered" structure (Yeagle et al., 1990) may incorporate into and traverse the membrane more readily than endogenous cholesterol. Thus, the calculated $t_{1/2}$ for cholestatrienol incorporation may be the lower limit for this process. Other studies also employing fluorescence techniques have determined cholesterol migration rates of approximately 10 min in red blood cells (Schroeder & Nemecz, 1990), mouse synaptosomes (Wood et al., 1989), and mouse LM fibroblasts (Schroeder & Nemecz, 1990); vesicle exchange techniques have provided longer migration rates from less than 4 h to 18 days [for review, see Schroeder and Nemecz (1990)]. Thus, the kinetics of cholestatrienol exchange into platelets from donor vesicles and the subsequent translocation of the sterol from the outer to the inner membrane monolayer are consistent with published observations. Extending these results to cholesterol suggests that cholesterol translocation in platelets may be relatively slow in comparison to erythrocytes.

A large body of work, using spin-labeled probes, fluorescent probes, and enzymatic susceptibility, has documented a stimulus-dependent redistribution of phosphatidylserine, phosphatidylethanolamine, and perhaps phosphatidylcholine in platelet membranes exposed to stimuli of aggregation (Bevers et al., 1982; Basse et al., 1993; Zwaal et al., 1993; Williamson et al., 1995). Recently, Gaffet et al. (1995) have shown that, less than 2 min after platelet activation by A23187, 50% of the phosphatidylserine in platelet plasma membrane was redistributed to the outer leaflet. This new distribution of phosphatidylserine was found to remain stable for 40 min. The redistribution of phosphatidylcholine and spingomyelin ($t_{1/2}$ 10 min) was found to be much slower (Gaffet et al., 1995). The results presented in this paper extend these observations by showing a redistribution of membrane cholesterol in platelets within 2 min of activation by collagen. In our studies, nearly 50% of the cholestatrienol was found to redistribute from the outer to the inner membrane leaflet. The new distribution of cholestatrienol in the membrane then remained stable for the next 30 min although after 40 min of incubation the distribution of the cholestatrienol appears biphasic, perhaps due to the reestablishment of the prestimulatory state of cholestatrienol distribution (Figure 3). The distribution of endogenous cholesterol, as inferred from cholesterol oxidase studies, also appeared to be altered in collagen-stimulated platelets. Collagen induced the movement of NBD-cholesterol out of the outer monolayer, but the movement of this probe was somewhat slower than cholestatrienol or endogenous cholesterol, and this maybe due to the bulky headgroup on this sterol probe. Alternatively, the membrane environment of the NBD-cholesterol (i.e., cholesterol rich vs cholesterol poor domain) may influence the kinetics of its redistribution. Under all of the experimental conditions tested, the redistribution of cholesterol was not accompanied by the appearance of fluorescent sterol in the incubation media, and both fluorescent probes (NBD cholesterol and cholestatrienol) remained localized to the plasma membranes after stimulation.

Collagen stimulation of platelets also resulted in the translocation of NBD-PE from the inner to the outer monolayer of the platelet membrane bilayer. This observation confirms work by others (Schroit & Zwaal, 1991; Schick et al., 1976; Bevers et al., 1982; Cha et al., 1977; Basse, 1993; Gaffet et al., 1995) employing spin-labeled probes and stimulus-dependent changes in phospholipase susceptibility showing the translocation of both phosphatidylethanolamine and phosphatidylserine across the platelet membrane monolayers. The translocation of NBD-PE in our study was not detected until 5 min of incubation with collagen. Gaffet et al. (1995) reported the translocation of a spin-labeled probe of phosphatidylserine in platelets occurring in less than 2 min. The more rapid translocation reported by Gaffet et al. (1995) may result from the use of A23187 as the platelet stimulus. A23187 is a more potent platelet stimulus than collagen and can promote the shedding of membrane vesicles and otherwise cause large imbalances in the membrane phospholipids. In previous studies using thrombin or thapsigarin as platelet activators, Smeets et al. (1993) reported a rate of phosphatidylserine outflux slower than that found by Gaffet et al. (1995). Alternatively, the bulky NBD group could retard the redistribution of this lipid.

The temporal relationship between cholesterol and NBD-PE movement and a consideration of the molecular structure and properties of these two lipids suggests a link between the translocation of these two lipids observed in these studies. Interestingly, cholesterol translocation, but not NBD-PE translocation, was blocked by the chaotropes urea and guanidine HCl. The translocation of cholesterol may be related to the translocation of PE through an ATP-dependent protein process, as would be the case if the translocation of PE was mediated by a reverse translocase. In this case, decreasing the ATP level would inhibit PE translocation (a translocase dependent lipid) (Devaux, 1990) but would not alter cholesterol translocation. This was not the case, however, as both PE and cholestatrienol translocation were inhibited with a decrease in ATP. Alternatively, PE and cholesterol translocation may be linked through a protein-independent process (i.e., a thermodynamic process). In this case, the link between these two lipids could be uncoupled with agents that stabilize PE and cholesterol within the same monolayer. This is indeed the case with the chaotropes urea and guanidine HCl. The results presented herein are consistent with model membrane (Yeagle, 1987; Backer & Dawidowicz, 1990) and biological membrane studies (Op den Kamp, 1979; House, et al., 1989) suggesting that the introduction of phosphatidylethanolamine into membranes creates a thermodynamically unfavorable environment for cholesterol. This effect is proposed to be due to the increased ordering of water molecules on the membrane surface. Chaotropic agents relieve the constraint on the membrane to conform to a nonbilayer-like structure by disrupting the interstitial hydrogen bonded water and thereby allow both phosphatidylethanolamine and cholesterol to coexist within the same monolayer. The possibility that the chaotropic agents act by blocking the activity of a sterol carrier protein cannot be rigorously excluded (Hapala et al., 1994). Since both collagen- and ADP-mediated cholestatrienol translocation is inhibited by chaotropes, the mechanism of action of these reagents is not due to a denaturation of the stimulant.

The experimental observations described in this paper emphasize the importance of platelet membrane surface properties. Upon stimulation of platelets by various aggregatory agents, the phospholipid asymmetry of this cell is disrupted in a fashion that appears to be irreversible. The translocation of phosphatidylserine is involved in the forma-

tion of the prothrombinase complex, thereby allowing further aggregation (Bevers et al., 1982). The translocation of phosphatidylethanolamine may also serve an essential role in platelet responses, specifically as a means by which the extracellular surface of the platelet may dehydrate. Such a dehydration is a necessary requirement for cell−cell adhesion (Leckband et al., 1993) and in some systems cell fusion (Bentz & Ellens, 1988). The heretofore unrecognized redistribution of cholesterol within platelet membrane may be necessary for the platelets to change shape (i.e., filopodia formation). Such membrane structures require a small radii of curvature, a structure more readily achieved in a PE-rich cholesterol poor region of the bilayer. The time scale of cholestatrienol redistribution is consistent with this possibility, since shape change is one of the early events of platelet aggregation. Alternatively, cholesterol redistribution may be important in the signaling pathway, given that cholesterol is one of the main determinants of platelet sensitivity to stimuli of aggregation (Shattil et al., 1975).

In more general terms, a number of transmembrane proteins are localized to either sterol-rich domains, as is the case with acetylcholine receptors (Fong & McNamee, 1986; Narayanaswami & Mc Namee, 1993) or require cholesterol for function (Yeagle, 1989; Craido et al., 1982; Carvalho et al., 1974). A redistribution of cholesterol between the two monolayers of a cell membrane would alter the dynamic properties of the membrane monolayers and could thereby impart a directionality in the control of cellular processes.

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