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COLLAGEN-STIMULATED UNIDIRECTIONAL TRANSLOCATION OF CHOLESTEROL IN HUMAN PLATELET MEMBRANES

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Summary

When human platelets are stimulated with collagen or thrombin, the asymmetric distribution of membrane lipids is disrupted as phosphatidylserine and phosphatidylethanolamine translocate from the inner monolayer to the outer monolayer. Coincident with the stimulus-dependent rearrangement of membrane phospholipids is a rapid redistribution of cholesterol from the outer to the inner membrane monolayer. This redistribution of cholesterol was observed when the stimulus was collagen or ADP. The data presented here show that epinephrine stimulation does not promote cholesterol translocation but does potentiate collagen-promoted movement of cholesterol. To investigate the process of cholesterol translocation, experiments were performed to determine whether collagen stimulated reverse cholesterol movement; i.e. from the inner to the outer monolayer. For this study, the fluorescent sterol cholestatrienol (C-3) was incorporated into platelet membranes by exchange from cholesterol-containing phosphatidylcholine small unilamellar vesicles. C-3 was then removed selectively from the outer monolayer by treatment of the platelets with bovine serum albumin (BSA). During the subsequent incubation of BSA-treated platelets, C-3 moved spontaneously into the outer from the inner monolayer. This translocation had an apparent half-time of approximately 25 min and was unaltered by the presence of collagen. These results suggest that collagen treatment of platelets selectively facilitates the inward movement of the sterol. We have hypothesized that cholesterol translocation may be thermodynamically driven as a result of an unfavorable entropy, resulting in cholesterol translocation out of an environment becoming enriched in phosphatidylethanolamine. The unidirectional nature of collagen-promoted cholesterol movement from the phosphatidylethanolamine-rich outer monolayer is consistent with this interpretation.

Key words: membrane lipid, translocation, cholesterol, platelet, human, collagen, epinephrine.

Introduction

The phospholipids of plasma membranes are distributed asymmetrically: the choline-containing lipids phosphatidylcholine and sphingomyelin constitute the majority of lipids in the outer monolayer, while ethanolamine and serine phospholipids are found predominantly in the inner monolayer (Op Den Kamp, 1979). This asymmetric distribution of phospholipids is maintained principally by the actions of an ATP-dependent translocase that transfers phosphatidylserine and phosphatidylethanolamine from the outer monolayer to the inner monolayer (Devaux, 1991). Some cells, such as platelets and erythrocytes (Zwaal and Schroit, 1997), have cellular processes that disrupt the asymmetric distribution of membrane lipids, either by causing the selective exposure of phosphatidylserine on the outer monolayer (Sune et al., 1987) or by effecting a scrambling of all membrane lipids (Williamson et al., 1995). The exposure of phosphatidylserine is required for catalysis of coagulation reactions (Bevers et al., 1982) and mediates recognition and uptake by phagocytes (Fadok et al., 1992).

The other major lipid constituent of cell membranes is cholesterol. Like phospholipids, cholesterol also appears to be distributed asymmetrically within those plasma membranes that have been investigated (Devaux, 1991; Schroeder et al., 1996; Dawidowicz, 1987). The non-uniform distribution of cholesterol is manifest as transbilayer cholesterol domains and as lateral cholesterol domains (Schroeder et al., 1996). The determinants of the non-uniform distribution of cholesterol and the physiological role subserved by the cholesterol domains are unresolved, although the strong possibility exists that such cholesterol domains are intimately involved with processes such as cholesterol trafficking and the cellular actions of cholesterol (Schroeder et al., 1996).

A compelling body of literature exists documenting the existence of phospholipid and cholesterol domains in biological membranes (Devaux, 1991; Dawidowicz, 1987; Schroeder et al., 1996). The physiological role subserved by lipid domains in platelets is of considerable interest for at least

two reasons. First, phospholipids in platelet plasma membranes undergo a rapid and dramatic reorganization in response to strong stimuli for aggregation. The mechanisms underlying phospholipid movement have been investigated, and two enzymatic activities have been identified: an ATP-dependent translocase, which transfers amine-containing phospholipids from the outer to the inner membrane monolayer (Devaux, 1991), and a Ca^{2+}-dependent scramblase (Comfurius et al., 1996; Zhou et al., 1997), which disrupts the asymmetric arrangement of phospholipids in platelets and erythrocytes upon cell stimulation. A phospholipid scramblase has been isolated from erythrocytes (Basse et al., 1996) and platelets (Comfurius et al., 1996) and found to mediate a Ca^{2+}-dependent bi-directional movement of phospholipids when constituted into phospholipid vesicles (Comfurius et al., 1996). The enzymatic activity is associated with a 37 kDa integral membrane protein; its cDNA cloning and deduced structure have been reported (Zhou et al., 1997), and its stable transfection into Raji cells normally expressing very low levels of the scramblase increased the exposure of phosphatidylserine on the outer membrane monolayer (Zhao et al., 1998).

Cholesterol exerts a profound influence upon platelet function. Platelets that are enriched with the sterol are dramatically more responsive to stimuli of aggregation than are platelets containing lower levels of cholesterol (Carvalho et al., 1974; Opper et al., 1995; Heemskerk et al., 1995; Schimmel et al., 1997). This action of cholesterol has important physiological consequences in terms of the role of cholesterol in the pathogenesis of arteriosclerosis (Carvalho et al., 1974). Despite the key role played by cholesterol as a determinant of platelet function, few studies of membrane cholesterol domains have employed platelets. We have investigated the membrane distribution of cholesterol in human platelets using the fluorescent cholesterol analogs NBD-cholesterol and $\Delta^{5,7,9,(11)}$cholestatrien-3β-ol (cholestatrienol; C-3) (Boesze-Battaglia et al., 1996). Our data showed that C-3 incorporates into platelet membranes with a half-time ($t_{1/2}$) of 39 min, with approximately 65 % of the probe partitioning into the outer monolayer. Upon stimulation of platelets with collagen or ADP, cholesterol translocated out of the outer monolayer into the inner monolayer. This translocation was closely associated with the exposure of phosphatidylethanolamine on the outer monolayer. We proposed that cholesterol translocation was governed by thermodynamic considerations. The present experiments were undertaken to study the directionality of basal and collagen-stimulated cholesterol translocation between the monolayers of the platelet membrane by measuring the reverse movement of cholesterol; i.e. out of the inner monolayer into a cholesterol-poor outer monolayer. Evidence is presented supporting the view that stimulus-dependent cholesterol translocation is unidirectional, an observation consistent with that of phosphatidylserine movement (Zwaal and Schroit, 1997). We also report that epinephrine, a less potent platelet activator, does not promote the redistribution of cholesterol.

Materials and methods

Platelet isolation and additional assays

Platelets were isolated from platelet-rich plasma obtained from the American Red Cross. The red blood cells were removed from the platelet-rich plasma by centrifugation at 750 revs min^{-1}, and the platelets were collected by centrifugation at 2500 revs min^{-1} for 20 min. The platelets were resuspended in a buffer containing NaCl (145 mmol l^{-1}), KCl (5 mmol l^{-1}), $MgSO_4$ (1 mmol l^{-1}) and Hepes (10 mmol l^{-1}), at pH 7.4, designated as platelet buffer. The platelet suspension was filtered on a column of 40 ml of Sepharose 4B (Pharmacia) to remove remaining plasma (Carvalho et al., 1974). The eluted platelets were centrifuged at 750 revs min^{-1} to remove any residual erythrocytes and suspended in platelet buffer. A sample of the platelet suspension was removed for assay of cholesterol (Allain et al., 1974) and phospholipid-phosphate (Bartlett, 1959).

Incorporation of fluorescent probes

These studies used cholestatrienol (C-3), which is structurally similar to cholesterol (Fig. 1) and which mimics cholesterol behavior in cell membranes (Schroeder and Nemecz, 1990; Yeagle, 1989a,b). This sterol is efficiently incorporated into cell membranes and into unilamellar vesicles and yields similar bilayer distribution and exchange rates to those obtained from experiments using [^{3}H]cholesterol (Yeagle, 1989a,b). C-3 (generously supplied by Dr Philip Yeagle) was incorporated into platelet membranes using small unilamellar vesicles prepared from phosphatidylcholine (egg) (Avanti Polar Lipids), cholesterol and C-3. The lipids were dissolved in acetonitrile, dried under nitrogen, lyophilized to remove trace amounts of solvent, dispersed in platelet buffer and sonicated to clarity. Large and multilamellar vesicles were removed by centrifugation at 45 000 revs min^{-1} for 30 min (Barenholtz et al., 1977). The cholesterol concentration of these exchange vesicles was 0.40 mol cholesterol mol^{-1} phospholipid, a value selected so as not to enrich or deplete the platelet membrane of endogenous cholesterol. The C-3 concentration of the small unilamellar vesicles did not exceed 2 mol %, relative to phospholipids. C-3 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 revs min^{-1} for 15 min, resuspended in platelet buffer and centrifuged a second time.

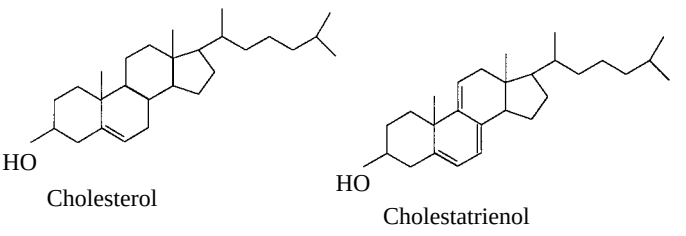

Fig. 1. Structural representation of cholesterol and cholestatrienol.

Fluorescence measurements

All fluorescence measurements were performed on a Perkin Elmer LS5B spectrofluorometer. Prior to the start of each experiment, $CaCl_2$ was added to the platelet suspension to a final concentration of 0.10 mmol l^{-1}, and the platelets were transferred to cuvettes placed in a water-jacketed turret at 37 °C or at 25 °C. C-3 fluorescence was measured at 390 nm with excitation at 324 nm. To determine the intramembrane distribution of C-3, its fluorescence was quenched with 2,4,6-trinitrobenzenesulfonic acid (TNBS). The initial fluorescence of the platelet suspension (designated as F_0) was recorded; various concentrations of TNBS (10–50 μmol l^{-1}) were then added until the fluorescence had decreased to a constant value. Triton X-100 was then added, causing a further decrease in fluorescence such that essentially all of the remaining fluorescence associated with C-3 was quenched. TNBS-quenchable C-3 fluorescence was calculated as a percentage of total fluorescence (F_0) and taken to represent that portion of the probe residing in the outer monolayer (McIntyre and Sleight, 1991). The fluorescence intensities were corrected for the background fluorescence measured on unlabeled platelets, which did not change during the course of the experiments, and for fluorescence associated with the added TNBS. C-3 fluorescence was quenched by energy transfer and confirmed as a shift in fluorescence intensity (Schroeder et al., 1979).

In some experiments, C-3 that had been incorporated into platelet membranes was removed by sequestration onto bovine serum albumin. Platelets were incubated with defatted bovine serum albumin (2 % w/v) at 37 °C for 5 min. The platelets were recovered by centrifugation, resuspended in platelet buffer and centrifuged a second time. Following treatment with bovine serum albumin, fluorescence attributed to C-3 decreased by approximately 50 %, and the percentage of platelet fluorescence quenched with TNBS decreased from 50 % to 14 % (Table 1). Coincident with the reduction in total C-3 fluorescence in platelets and the percentage of platelet fluorescence quenched by TNBS, there occurred a decrease of slightly more than 40 % in total platelet cholesterol content. These observations suggest that incubation with BSA selectively removes only that portion of the fluorescent probe and sterol that are distributed in the outer monolayer of the platelet membrane.

Results

We have reported previously (Boesze-Battaglia et al., 1996) a rapid redistribution of cholesterol from the outer leaflet of the platelet plasma membrane into the inner leaflet following stimulation of the cells with collagen or ADP. The experiment presented in Fig. 2 sought to determine whether epinephrine would also promote an intramembrane redistribution of cholesterol. In this study, between 47 % and 54 % of the fluorescence of C-3 was quenched by the addition of TNBS, suggesting that approximately 50 % of the C-3 was present in the outer monolayer. Exposure of platelets to epinephrine (10 μmol l^{-1}) did not affect the accessibility of the probe to TNBS. In contrast, when platelets were exposed to collagen,

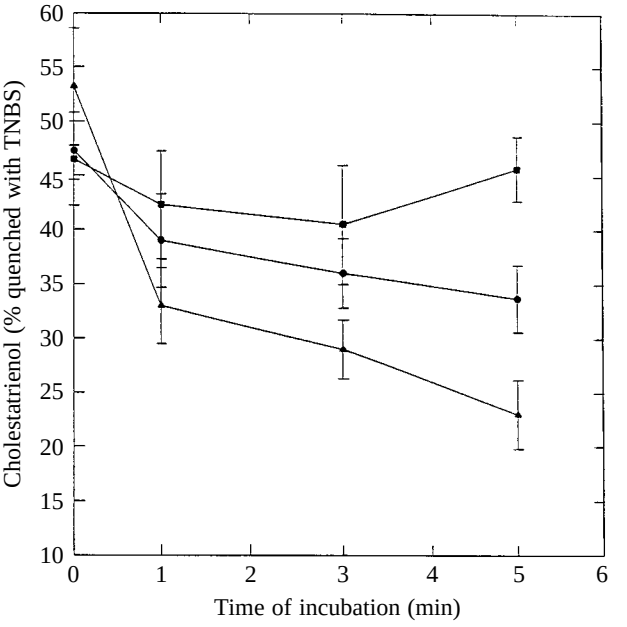

Fig. 2. Effects of collagen and epinephrine, singly and in combination, on the accessibility of cholestatrienol to quenching with 2,4,6-trinitrobenzenesulfonic acid (TNBS). Platelets were loaded with cholestatrienol by incubation with small unilamellar vesicles. Following incubation, cholestatrienol-loaded platelets were incubated with 10 μg ml^{-1} collagen (●), with 10 μmol l^{-1} epinephrine (■) or with 10 μg ml^{-1} collagen and 10 μmol l^{-1} epinephrine (▲) for the times given on the abcissa. The amount of cholestatrienol in the outer monolayer is determined as the decrease in cell-associated fluorescence after quenching with TNBS and is calculated as described in Materials and methods. Each value is the mean and standard error of four separate experiments, each performed on a different platelet preparation.

the percentage of C-3 accessible to TNBS decreased significantly ($P<0.05$) to less than 40 %, a result interpreted to reflect movement of cholesterol out of the outer monolayer (Boesze-Battaglia et al., 1996; McIntyre and Sleight, 1991). This finding indicates that epinephrine, unlike collagen, does not cause a redistribution of membrane cholesterol.

When epinephrine and collagen were present simultaneously, however, the percentage of the probe quenched by TNBS decreased to values significantly ($P<0.05$) lower than that observed when collagen was present alone. This finding suggests that, while epinephrine by itself is without influence upon the intramembrane distribution of cholesterol, it did enhance the collagen-dependent movement of the sterol out of the outer monolayer. This finding is consistent with other reported actions of epinephrine on platelets to potentiate responses elicited by agonists more potent than epinephrine while not directly stimulating aggregation (Lanza et al., 1988).

The data shown in Fig. 2 confirm our previous finding (Boesze-Battaglia et al., 1996) that collagen promotes a redistribution of cholesterol from the outer monolayer into the inner monolayer. We proposed that cholesterol movement

Table 1. *Platelet cholesterol and cholestatrienol fluorescence following treatment with bovine serum albumin*

Treatment	Platelet [cholesterol] (mol mol^{-1} P_i)	Total fluorescence	Fluorescence after TNBS	Percentage quenched
None	0.30±0.04	72.9±10.9	34.5±12.1	52.1±3.8
Bovine serum albumin	0.14±0.02	37.6±7.5	32.5±8.1	13.5±5.1

Platelets were loaded with cholestatrienol by incubation with small unilamellar vesicles as described in Materials and methods. Following incubation, cholestatrienol-loaded platelets were incubated with platelet buffer or with platelet buffer containing 2% bovine serum albumin (w/v) for 5 min at 37 °C. Platelets were recovered by centrifugation and washed once with platelet buffer. Fluorescence was recorded before and after addition of trinitrobenzenesulfonic acid (TNBS) and is expressed in arbitrary units. Cholesterol was assayed enzymatically and expressed with respect to the platelet phospholipid phosphate concentration. The data presented above are the fluorescence values following subtraction of the value obtained from unloaded platelets (83 in control cells and 86 in cells treated with bovine serum albumin).

Each value is the mean ± S.E.M. of six separate experiments, each performed on a different platelet preparation.

within platelet membranes is mediated by the relative content of phosphatidylethanolamine in the membrane monolayer (Boesze-Battaglia et al., 1996). To investigate this proposal further, we investigated whether the exposure of platelets to collagen would promote a directional movement of cholesterol from the inner to the outer monolayer; i.e. in the opposite direction. To detect reverse cholesterol translocation from the inner to the outer monolayer, platelets were labeled with C-3 as described above and then incubated with fatty-acid-free bovine serum albumin to remove the fluorescent sterol from the outer monolayer (see Table 1). When albumin-treated platelets were reincubated, the percentage of TNBS-quenchable fluorescence gradually increased from less than 10% to more than 50% after 30 min of incubation (Fig. 3). Over the same period, the total platelet fluorescence did not change: the C-3 fluorescence calculated to be present in the inner monolayer decreased progressively during the incubation. These observations are consistent a redistribution of the sterol from an inner monolayer to an outer monolayer, secondary to the removal of sterol from the outer monolayer.

We next sought to determine whether collagen altered the movement of C-3 from the inner to the outer monolayer. This question was posed in view of our finding that cholesterol redistribution out of the outer monolayer was promoted by collagen. If collagen-stimulated cholesterol movement was based purely on mass action, then we would expect to find the reverse movement of the sterol to be stimulated by collagen as well. The data in Fig. 4 show this not to be the case. Fig. 4 depicts the results of an experiment in which C-3-labeled platelets were incubated with BSA to sequester outer-monolayer C-3 and then reincubated in the absence or presence of collagen. The presence of collagen did not accelerate the reintroduction of C-3 into the outer monolayer. To the contrary, collagen tended to retard the redistribution of C-3 into the outer monolayer during the first 7.5 min of incubation. This may reflect, on the basis of previous data (Boesze-Battaglia et al., 1996), the stimulation by collagen of the movement of any residual outer-monolayer cholesterol into the inner monolayer, thereby resulting in an apparent 'lag' time associated with reverse cholesterol movement. Coincidentally, the slowing of reverse cholesterol movement may result from the stimulation of phosphatidylethanolamine movement into the outer leaflet following collagen exposure.

The absence of collagen stimulation of reverse C-3 movement was consistent with our suggestion (Boesze-

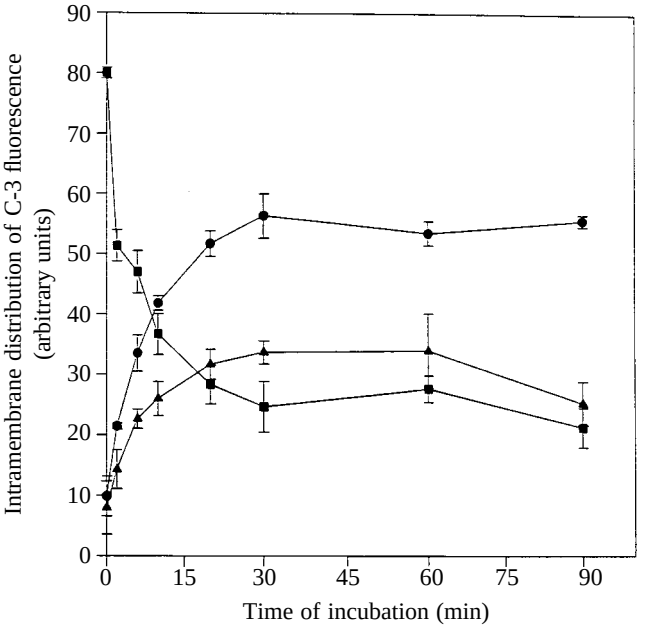

Fig. 3. Movement of cholestatrienol from the inner monolayer into the outer monolayer. Platelets were loaded with cholestatrienol by incubation with small unilamellar vesicles. Following incubation, cholestatrienol-loaded platelets were incubated with platelet buffer containing 2% bovine serum albumin (w/v) for 5 min at 37 °C. Platelets were recovered by centrifugation, washed once with platelet buffer and reincubated at 37 °C for the times given on the abscissa. Fluorescence was recorded before and after addition of 2,4,6-trinitrobenzenesulfonic acid (TNBS). The amount of cholestatrienol in the outer monolayer is determined as the decrease in cell-associated fluorescence after quenching with TNBS and is expressed either as the corrected fluorescence value (▲) or as a percentage of total platelet fluorescence (●). The amount of cholestatrienol in the inner monolayer (■) is calculated from the fluorescence remaining after quenching with TNBS. Each value (in arbitrary units) is the mean and standard error of four separate experiments, each performed on a different platelet preparation.

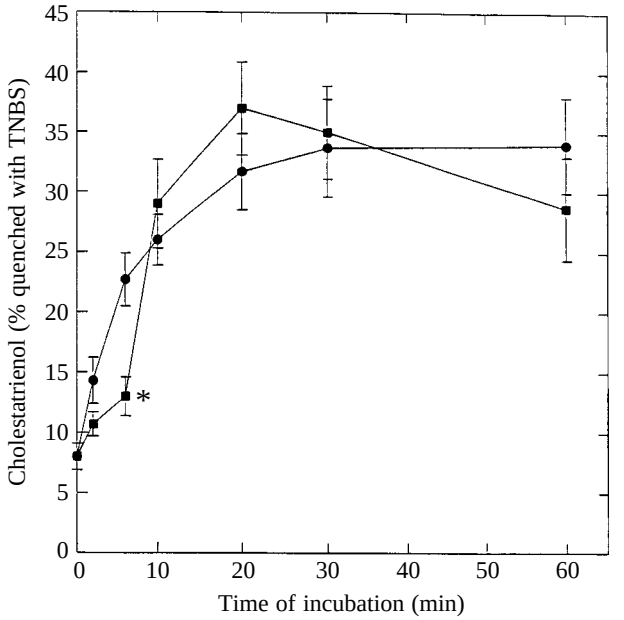

Fig. 4. Collagen does not stimulate cholestatrienol movement from the inner to the outer monolayer of platelet membranes. Platelets were loaded with cholestatrienol by incubation with small unilamellar vesicles. Following incubation, cholestatrienol-loaded platelets were incubated with platelet buffer containing 2% (w/v) bovine serum albumin for 5 min at 37 °C. Platelets were recovered by centrifugation, washed once with platelet buffer and reincubated at 37 °C for the times given on the abscissa in the absence (●) or presence (■) of 10 μg ml^{-1} collagen. Fluorescence was recorded before and after addition of 2,4,6-trinitrobenzenesulfonic acid (TNBS). The amount of cholestatrienol in the outer monolayer is determined as the decrease in cell-associated fluorescence after quenching with TNBS and is calculated as described in Materials and methods. Each value is the mean and standard error of four separate experiments, each performed on a different platelet preparation. *Significantly different from control value (P<0.05).

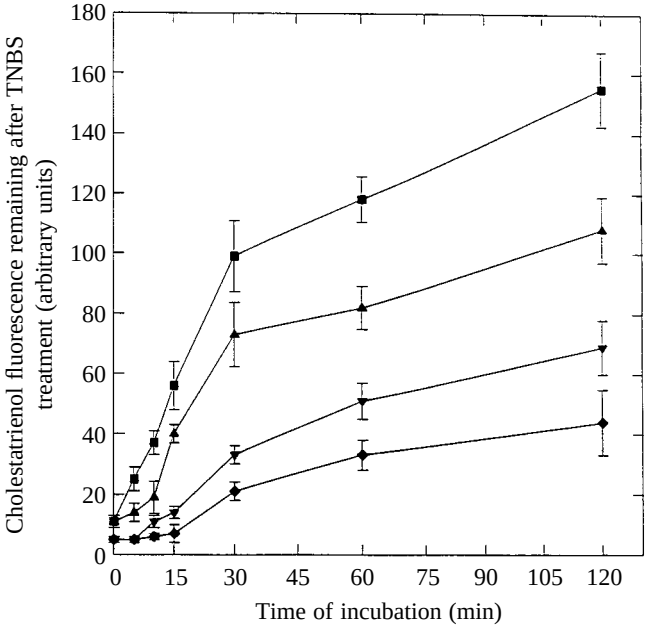

Fig. 5. Effect of collagen on the uptake of cholestatrienol into platelet membranes at 25 °C and at 37 °C. Cholestatrienol incorporation from small unilamellar vesicles into platelet membranes was measured at 25 °C (▼,◆) or at 37 °C (▲, ■) in the absence (▲, ◆) or in the presence (■, ▼) of collagen. Each fluorescence value was corrected for the intrinsic fluorescence associated with a platelet suspension not loaded with cholestatrienol. The data are expressed as the total fluorescence associated with the inner platelet monolayer and are taken to represent the residual value following quenching with 2,4,6-trinitrobenzenesulfonic acid (TNBS). Collagen was present at a final concentration of 10 μg ml^{-1}. Each value shown is the mean and standard error of three experiments, each performed on a different platelet preparation.

Battaglia et al., 1996) that the process of stimulus-dependent sterol redistribution was unidirectional because of the concomitant translocation of phosphatidylethanolamine into the outer monolayer. To obtain additional evidence for this possibility, the incorporation of C-3 into the inner membrane monolayer was measured at two different temperatures. The data shown in Fig. 5 show incorporation of C-3 into the inner monolayer of platelet membranes at 37 °C and 25 °C. At both temperatures, the presence of collagen increased the incorporation of probe into the inner monolayer. Although substantially less probe was incorporated at 25 °C than at 37 °C, the percentage of incorporated probe that was not accessible to TNBS quenching and can, therefore, be deduced to reside in the inner monolayer was nearly identical (at 37 °C, 55% accessible to TNBS; at 25 °C, 51% accessible to TNBS) at the two temperatures. In addition, the calculated rates, measured over the entire incubation period, for C-3 uptake into the inner monolayer at the two incubation temperatures were remarkably similar (0.045 fluorescence units min^{-1} at 25 °C and 0.050 fluorescence units min^{-1} at 37 °C).

Fig. 6 shows a kinetic analysis of C-3 reincorporation into the outer monolayer using the data shown previously in Fig. 3. The $t_{1/2}$ of this process was estimated to be approximately 25 min from a plot of $\log_e(F_t/F_0)$, where F_t is the fluorescence at time t and F_0 is the fluorescence at time zero, *versus* time and the rate was estimated to be 0.028 fluorescence units min^{-1}. The kinetics of reverse cholesterol movement are similar to those for uptake of C-3 into platelet membranes and for the selective incorporation of probe into the inner monolayer (Fig. 5).

Discussion

While considerable effort has been spent on defining and identifying the functional role of a unidirectional translocation of phosphatidylserine, very few experimental data are available describing a functional consequence for the intramembrane distribution of a potent platelet modifier cholesterol. Previous studies from this laboratory have utilized the fluorescent probe C-3 to investigate the intramembrane distribution of cholesterol in human platelets. We reported a rapid stimulus-dependent redistribution of cholesterol from the outer to the

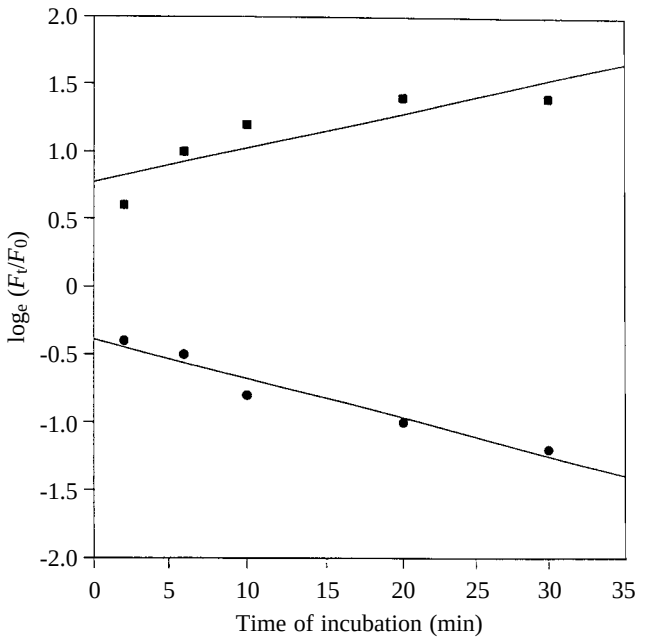

Fig. 6. Kinetic analysis of cholestatrienol movement into the outer monolayer from the inner monolayer of platelet membranes following incubation with bovine serum albumin (BSA). Platelets were loaded with cholestatrienol by incubation with small unilamellar vesicles. Following incubation, cholestatrienol-loaded platelets were incubated with platelet buffer containing 2% (w/v) BSA for 5 min at 37 °C. Platelets were recovered by centrifugation, washed once with platelet buffer and reincubated at 37 °C for the times given on the abscissa. Fluorescence was recorded before and after addition of 2,4,6-trinitrobenzenesulfonic acid (TNBS) and corrected for the intrinsic fluorescence associated with a platelet suspension not loaded with cholestatrienol. The data are given as the natural logarithm ($\log_e$) of the ratio of each corrected fluorescence value at the indicated incubation times to the fluorescence at time zero. The line designated with the filled circles (●) represents the fluorescence remaining after quenching with TNBS (inner monolayer); the line designated with the filled squares (■) represents the fluorescence quenched with TNBS (outer monolayer). The slopes of the calculated lines are used to derive half-times ($t_{1/2}$) of cholestatrienol movement of 27.4 out of the inner monolayer and 24.2 min into the outer monolayer.

inner monolayer (Boesze-Battaglia et al., 1996). In the present study, we sought to determine the directionality of cholesterol translocation and whether cholesterol translocation in the opposite direction (i.e. from the inner monolayer to the outer monolayer) was stimulus-dependent. In our translocation assays, the platelet outer membrane was depleted of cholesterol and the fluorescent probe C-3 by incubation with defatted albumin. Upon reincubation, a portion of the C-3 fluorescence became increasingly susceptible to quenching with TNBS. This finding is interpreted as the spontaneous translocation of C-3 and cholesterol from the inner into the outer monolayer. This interpretation is supported by the similarity between the kinetics of movement of this sterol and the kinetics of uptake of C-3 into platelet membranes (Fig. 5 and Boesze-Battaglia et al., 1996). We have previously shown that the movement of C-3 is kinetically similar to the movement of cholesterol in platelets and occurs on the time scale of minutes as described previously for brush-border membrane (el Yandouzi and Le Grimellec, 1992). When collagen was added, however, the apparent movement of cholesterol out of the inner monolayer and into the outer monolayer was not stimulated; instead, collagen appeared to retard slightly the reintroduction of cholesterol probe into the outer monolayer. Thus, the ability of collagen to promote cholesterol redistribution within the platelet plasma membrane appears to be restricted to promoting the movement of cholesterol out of the outer membrane monolayer.

The cellular-biochemical factors that govern the intramembrane distribution of cholesterol in platelet membranes have not been rigorously established. As is the case with phospholipids, cholesterol is distributed non-uniformly within cell membranes (Schroeder et al., 1996; Dawidowicz, 1987). Transbilayer cholesterol domains may be determined by alterations in the rates of sterol translocation across the membrane or by alterations in the equilibrium values of cholesterol distribution (Schroeder et al., 1996). Platelets may be unique in that the membrane cholesterol in these cells redistributes as a consequence of stimulation with aggregatory agents (Boesze-Battaglia et al., 1996). The mechanisms underlying this stimulus-dependent cholesterol redistribution have not been established, although one possibility is that a membrane protein, similar to the acyl transferases (Devaux, 1991) or the phospholipid scramblase (Comfurius et al., 1996), mediates this response. Indeed, two cytosolic cholesterol-binding proteins have been identified which affect sterol transfer among intracellular and plasma membranes (Schroeder et al., 1993; Frolov et al., 1996). These proteins have not yet been identified in platelets, and their possible role in redistributions of cholesterol within cell membranes has yet to be investigated. However, the transfection of fibroblasts with cDNA coding for one of the sterol-binding proteins was found to lower the inner membrane monolayer content of cholesterol (Woodford et al., 1993).

We proposed that cholesterol movement within platelet membranes is mediated by the relative content of phosphatidylethanolamine in the membrane monolayers (Boesze-Battaglia et al., 1996). In this model, the presence of phosphatidylethanolamine in membranes creates a thermodynamically unfavorable environment for cholesterol, causing the sterol to partition into a more favorable environment (Backer and Dawidowicz, 1981; House et al., 1989; Yeagle, 1987). In the absence of stimulation, cholesterol is localized in the phosphatidylethanolamine-poor outer membrane monolayer. Upon stimulation, phosphatidylethanolamine translocates from the inner to the outer membrane monolayer, creating a thermodynamically unfavorable environment for cholesterol. This hypothesis is consistent with our finding in the present study that reverse cholesterol movement is significantly retarded by collagen (Fig. 4), and this observation is most readily explained by the

concomitant stimulation by collagen of sterol translocation from the outer into the inner monolayer (Boesze-Battaglia et al., 1996). We also report a similar action of collagen at 25 °C and 37 °C promoting the incorporation of C-3 into the inner monolayer of platelet membranes (Fig. 5).

These findings are both consistent with the proposed role of phosphatidylethanolamine exerting an influence on cholesterol distribution since the outer monolayer is becoming enriched with this phospholipid as a result of collagen stimulation. The absence of collagen stimulation of reverse cholesterol movement (i.e. from inner to outer monolayer) and the finding that lowering the incubation temperature to 25 °C does not selectively block the accumulation of C-3 into the inner platelet membrane are consistent with the our hypothesis (Boesze-Battaglia et al., 1996) that cholesterol distribution in the membrane is dependent upon neighboring phospholipids. Phosphatidylethanolamine has also been suggested to play a role in determining the distribution of cholesterol between the disc and plasma membranes in photoreceptor rod cells. In rod cells, the internal disc membranes contain substantially less cholesterol than does the plasma membrane (Boesze-Battaglia and Albert, 1989). This is thought to result from the enrichment of disc membranes with phosphatidylethanolamine, which provides a thermodynamically less favorable environment for the sterol (Boesze-Battaglia and Schimmel, 1996; Yeagle, 1987; Yeagle, 1989a,b).

The biochemical mechanisms that account for collagen stimulation of cholesterol translocation have yet to be rigorously investigated. Collagen-induced translocation of phosphatidylethanolamine into the outer monolayer may result from increased activity of the phospholipid scramblase, which is stimulated by elevations in cytosolic Ca^{2+} concentration (Comfurius et al., 1996; Zhou et al., 1997). Whether the scramblase-induced enrichment of the outer monolayer with phosphatidylethanolamine is sufficient for the concomitant translocation of cholesterol, or whether another action of collagen unrelated to the scramblase is required, has yet to be established. Interestingly, epinephrine potentiates collagen-induced cholesterol translocation (Fig. 2) but does not affect cholesterol distribution by itself. Epinephrine alone does not induce an increase in cytosolic Ca^{2+} concentration (Lanza et al., 1988) and would not therefore be expected to activate the scramblase. However, epinephrine does potentiate the increase in cytosolic Ca^{2+} concentration caused by other agents, including collagen (Ardlie et al., 1987), and thus would be expected to potentiate collagen-enhanced lipid translocations in the platelet membrane.

The interest in cholesterol-rich and -poor lipid domains in platelets comes from examination of the considerable influence exerted by cholesterol on platelets. Platelets are incapable of *de novo* synthesis of cholesterol, and the total membrane cholesterol content of platelets is reflective of the plasma cholesterol level. Platelets harvested from hypercholesterolemic individuals have higher than normal membrane cholesterol levels and aggregate more readily upon stimulation (Carvalho et al., 1974; Shattil et al., 1977). Furthermore, platelets enriched with cholesterol by incubation with cholesterol-containing small unilamellar vesicles are more sensitive to aggregatory stimuli (Shattil et al., 1975; Heemskerk et al., 1995; Opper et al., 1995; Schimmel et al., 1997). The intramembrane distribution of cholesterol in platelets has not been investigated, and the possibility that the effects of cholesterol are exerted by specific cholesterol domains is an attractive hypothesis. Cholesterol translocation has been detected following platelet stimulation with collagen, ADP or thrombin but not, as shown in the present study, with epinephrine. The absence of epinephrine stimulation of cholesterol translocation is not surprising since epinephrine is a weaker stimulator of platelets. Epinephrine does appear to enhance the ability of collagen to promote cholesterol translocation, and other platelet functions also appear to be potentiated by epinephrine (Lanza et al., 1988). Interestingly, epinephrine-induced platelet aggregation is also enhanced by hypercholesterolemia (Carvalho et al., 1974).

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