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Microenvironment Changes of Human Blood Platelet Membranes Associated with Fibrinogen Binding

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Summary. Alterations in the membrane organization caused by fibrinogen binding to human blood platelets and their isolated membranes were analyzed by fluorescence and electron spin resonance measurements. The degree of fluorescent anisotropy of DPH, ANS and fluorescamine increased significantly when fibrinogen reacted with its membrane receptors. Both fluorescence and ESR analyses showed that fibrinogen binding to platelet membranes is accompanied by an increase of the membrane lipid rigidity. This effect seems to be indirect in nature and is mediated by altered membrane protein interactions. As it has been shown that an increased membrane lipid rigidity leads to a greater exposure of membrane proteins, including fibrinogen receptors, this might facilitate a formation of molecular linkages between neighboring platelets. On the other hand, changes of fluorescence anisotropy of membrane tryptophans and N-(3-pyrene) maleimide suggest the augmented mobility of the membrane proteins. Evidence is presented which indicated that the binding of fibrinogen to the membrane receptors is not accompanied by any changes in the fluorescence intensity of ANS attached to the membranes. It may suggest that the covering of platelets with fibrinogen does not influence the surface membrane charge. In contrast to fibrinogen, calcium ions caused an increase of the fluorescence intensity resulting from the more efficient binding of ANS to the platelet membranes.

Key Words fibrinogen · platelet membranes · spectroscopy · fluorescent probes

Introduction

The mechanism of the fibrinogen-platelet interaction has been recently broadly investigated but yet not completely understood (Marguerie, Plow & Edgington, 1979; Marguerie, Edgington & Plow, 1980; Niewiarowski et al., 1981). Specific saturable receptors for fibrinogen molecules present on the human platelet surface were found to be rapidly induced by ADP in the presence of Ca^{2+} (Nachman & Ferris, 1974; Marguerie et al., 1979). These receptors were active in the isolated platelet membranes as well (Cierniewski et al., 1982). It can be expected that the receptor induction might lead to disturbances in the organization of both the lipid and the protein components in the platelet membranes. Such rearrangements can be seemingly mediated by alterations in the fluidity of platelet membrane lipids. This was already observed by spin-label studies of platelets treated with Ca^{2+} and La^{2+} ions (Sauerheber et al., 1980). On the other hand a binding of fibrinogen may influence the membrane surface charge.

In principle, mobility of the fibrinogen receptors is controlled by the local microviscosity and plasticity properties of the membrane. They are determined mainly by the composition of the cell surface lipids and their distribution pattern. To follow microviscosity changes of the platelet membranes, the fluorescence analysis of the fluorescent probes may be a useful and sensitive technique (Cogan et al., 1973; Nathan et al., 1979, 1980; Liu et al., 1980; Steiner, 1981). The fluorescent probes can be embedded into different regions of the platelet membranes, i.e. in the surface lipid layer, in the lipid core, at the lipid-protein contacts or attached to the membrane proteins (Radda & Vanderkooi, 1972). Thus any changes in microviscosity of these membrane regions can be monitored by fluorescence properties of the attached probe. An independent estimation of these phenomena is possible using the spin-label ESR technique (Boggs, Vail & Moscarello, 1976; Gordon & Sauerheber, 1977; Sauerheber et al., 1980).

This study was an attempt to determine the very early effects of ADP and fibrinogen on the platelets' membrane mobility using the fluorescence and spin label techniques. To avoid large membrane changes associated with the platelet aggregation, experiments were usually performed in the absence of calcium which is known to be required for the efficient binding of fibrinogen to membrane receptors, a prerequisite of the ADP-stimulated platelet aggregation.

Abbreviations: ANS: 1-anilino-8-naphthalene sulfonate; DPH: 1,6-diphenyl-1,3,5-hexatriene; 3-PM: N-(3-pyrene)maleimide; I(12,3): nitroxide-labeled stearic acid; ADP: adenosine 5′-diphosphate; Fg: fibrinogen.

Materials and Methods

Chemicals

Pyrene, 1-anilino-8-naphthalene sulfonate (ANS), and 4-phenylspiro-furan-2(3H),-1′phthalan-3,3′-dione (fluorescamine) were obtained from Aldrich-Europe (Belgium) and 1,6-diphenyl-1,3,5-hexatriene (DPH) was from Syva Co. (Palo Alto, Calif.). N-(3-pyrene)maleimide (3-PM) was purchased from Fluka, and adenosine 5′-diphosphate, sodium salt (ADP) from Sigma Chemical Co. The spin label I (12,3), N-oxyl-4,4-dimethyloxazolidine derivative of 5-ketostearic acid was from Syva Co. All other chemicals were from Serva Feinbiochemica or from POCH, Poland. Fluorescence cuvettes and capillary pipettes were siliconized using silicon from Clay Adams, N.Y.

Preparation of the Washed Platelets

Platelets were isolated from human blood freshly collected in acid-citrate-dextrose (10:1) by differential centrifugation (Mustard et al., 1977) and gel filtration (Tangen, Berman & Marfey, 1971). Platelet-rich plasma (PRP) was obtained by a 20-min centrifugation at 200 *g* (1,000 rpm) and centrifuged for 20 min at 1000 *g* (4000 rpm) to sediment the platelets. The resulting pellet was resuspended in 8 ml of the modified Tyrode's buffer (140 mM NaCl, 5 mM KCl, 10 mM glucose and 15 mM Tris-HCl, pH 7.4), and the cells were subsequently washed three times by repeated centrifugation at 500 *g* for 10 min in the above buffer. Otherwise, the sedimented platelets were resuspended in 1 ml of platelet-poor plasma (PPP) and applied on a Sepharose 2 B column (1 × 30 cm), equilibrated with the modified Tyrodes buffer. The column was washed with the same buffer and the platelets were recovered in the void volume. The entire washing procedure was performed in the plastic and carried out at room temperature. Final concentration of the platelets was 2 to 5×10^9 cells per ml.

Preparation of the Platelet Membranes

Platelet membranes were isolated according to the method of Barber and Jamieson (1970). All buffers contained 10 mM benzamidine and 10 mM ε-amino caproic acid to prevent proteolytic degradation. Protein composition of the platelet membranes was the same as described previously (Cierniewski, Krajewski & Wodzinowska, 1976). Our previous studies (Cierniewski et al., 1982) showed no detectable difference in binding behavior between the lighter and heavier plasma membrane bands and, thus, the two bands were pooled for spectrofluorimetric analyses. Membrane suspension in 10 mM Tris-HCl buffer (pH 7.4) was diluted to the protein concentration of about 200 μg/ml.

Fibrinogen Isolation

The platelet-poor plasma was treated with barium sulfate to remove vitamin K-dependent factors, whereafter highly purified human fibrinogen was prepared by cold ethanol precipitation followed by ammonium sulfate fractionation at 26% saturation at 4 °C (Doolittle, Schubert & Schwartz, 1967). Protein concentration was determined spectrophotometrically (Mihalyi, 1968).

Labeling of the Platelet Membranes with Fluorescent Probes

Pyrene dispersion was prepared from chloroform solution at a concentration of 0.1 M. Small aliquots of the above solution were evaporated to dryness and 500 μl of the suspension of the platelets or platelet membranes were added to a final concentration of 0.5 mM of pyrene per 3×10^8 platelets or 210 μg of the membrane proteins. Lipid-to-pyrene molar ratio was 130:1 (Galla & Sackman, 1974). The mixtures were gently shaken and incubated at 37 °C for an hour. Measurements were carried out at room temperature in 400-μl cylindrical cuvettes.

ANS was diluted in the Tris-HCl buffer described above. Platelet suspension (2×10^9 cells per ml) was incubated for an hour at room temperature with 4 vol of 2.5 mM ANS solution (Hashimoto, Shibata & Kobayashi, 1977). Free ANS was removed by two cycles of washing with the above buffer. In the membrane investigation a small volume of a stock solution of ANS was added to the suspension of platelet membranes to a final concentration of 50 μM. Concentration of the platelet membrane protein was 150 μg/ml.

DPH (4×10^{-4} M) in dimethylformamide was diluted 100-fold with a vigorously stirred aqueous solution of Tris-HCl buffer (pH 7.4). After addition of an equal volume of membrane suspension (400 μg protein per ml) the mixture was incubated for 2 hr at 37 °C (Steiner, 1981).

Small aliquots of 3-PM dissolved in ethanol and fluorescamine in acetone were added to the final concentration of 50 or 500 μM, respectively. The membrane suspensions (200 μg of protein per ml) were incubated with the above fluorescent probes for an hour at room temperature.

Fluorescence Measurements

Fluorescence measurements were carried out in a Perkin-Elmer MPF-3L spectrofluorometer, fitted with polarizers of the excitation and emission paths as well as a thermostatically controlled cuvette holder. Excitation and emission wavelengths were: a) 320 and 378 or 470 nm for pyrene, b) 360 and 440 nm for DPH, c) 380 and 480 nm for ANS, d) 390 and 480 nm for fluorescamine, e) 345 and 380 nm for 3-PM and f) 295 and 333 nm for tryptophan fluorescence, respectively. In each experiment control samples of the fluorescent probe solutions and the unlabeled membranes were examined according to Radda (1975), and corrections for the scatter contribution (approx. 2 to 5% of the total fluorescence) were made.

For pyrene studies, the ratio $I_{470}:I_{378}$-fluorescence intensity of the excimer and monomer, respectively, was calculated from the emission spectra of the probe incoporated into the lipid region of the platelet membrane.

For fluorescence polarization studies fluorescence intensities were measured parallel and perpendicular to the direction of the polarized excitation beam. From these measurements the fluorescence polarization P and anisotropy r were calculated according to the equations:

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}},$$

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2\,GI_{VH}}$$

where I_{VV} and I_{VH} are the fluorescence intensities measured with a vertical polarizer and analyzer mounted vertically and horizontally, respectively. $G = I_{HV}/I_{HH}$ is the correction factor (Chen & Bowman, 1965).

The interrelationship of P and r is obvious, henceforth we employed only r parameter in this paper. Due to the technical limitations determination of the fluorescence lifetime of the probes in the platelet membranes could not be carried out. In the case of DPH the structural parameter of the Perrin equation could not be measured either (Inbar & Shinitzky, 1975). Thus the calculated values do not reflect absolute microviscosity of the lipid core and may serve for comparative purposes only.

Spin Labeling

For the spin-labeling studies 5 µl of the I (12,3) spin probe dissolved in ethanol (10 mg/ml) was first evaporated to dryness and then 100 µl of the platelet suspension (5×10^9 pl per ml) was added and gently vortexed for several minutes at room temperature.

Analysis of ESR Spectra

The ESR spectra of the I(12,3)-labeled platelets were recorded by a Varian E-3 ESR spectrometer with a 100 G (10 mT) field sweep and 8 min scan time. Time constant was 0.3 sec and amplitude modulation 2 G (0.2 mT). The relative position of the maxima or minima were used to determine the outer and inner hyperfine splitting ($T_{\|}$ and $T_{\perp}$). $2T_{\perp}$ was corrected by the addition of 1.6 G (Hubbell & McConnell, 1971). The inner and outer hyperfine splitting values permit to calculate an order parameter of the membrane lipids according to the equation:

$$S=\left(\frac{T_{\|}-T_{\perp}}{T_{zz}-T_{xx}}\right)\left(\frac{a_N}{a_{N'}}\right) \qquad \begin{aligned} a_N &= 1/3(T_{zz}+2T_{xx}) \\ a_{N'} &= 1/3(T_{\|}+2T_{\perp}) \end{aligned}$$

where $T_{\|}$ and $T_{\perp}$ = outer and inner hyperfine splitting, respectively, estimated from the experimental ESR spectra, and T_{zz} and T_{xx} = corresponding values for the perfectly ordered systems, i.e. 32.4 G and 6.1 G (Seelig, 1970).

ADP and Fibrinogen Binding Assays

Binding of fibrinogen (0.01 µM) to platelet membranes (200 µg proteins per ml) was performed in the presence and absence of ADP (0.15 µM), after a 20-min incubation (Cierniewski et al., 1982). Separately, the effect of ADP both on the platelet membranes and washed platelets (6 µM) per 10^8 pl) was studied, as well as the effect of fibrinogen (0.2 mg/10^8 pl) on washed platelets in the presence and absence of ADP.

Results

Pyrene Excimer Formation

Pyrene is an apolar optical probe well suited for the investigation of the lipid bilayer membranes. The pyrene molecule is incorporated into the hydrophobic region of the bilayer, as demonstrated by NMR spectroscopy (Vanderkooi et al., 1975). Measurements of the monomer and excimer fluorescence intensities and a single lifetime of pyrene allow to estimate its lateral diffusion coefficient (Galla & Sackman, 1974; Galla & Hartman, 1980; Galla & Luisetti, 1980). Fluorescence spectrum of pyrene embedded in human platelet membranes is

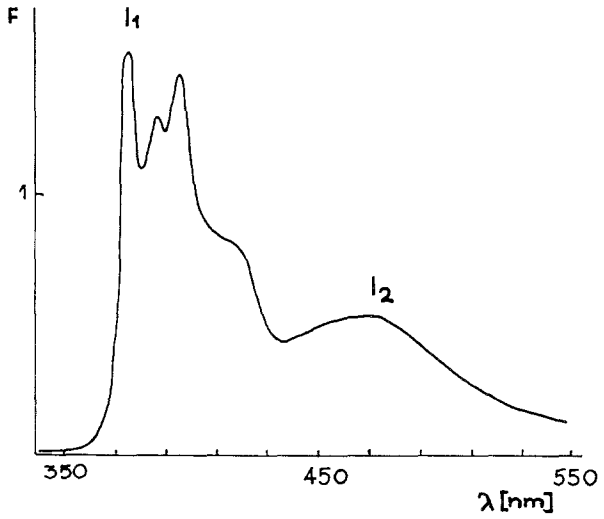

Fig. 1. Fluorescence emission spectra of pyrene in human blood platelets. $\lambda_{ex}=320$ nm. I_1 and I_2 indicate fluorescence intensity of monomer and excimer, respectively

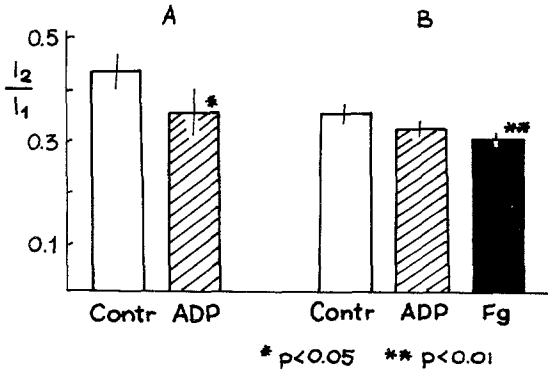

Fig. 2. Excimer to monomer intensity ratio (I_2:I_1) of pyrene in human blood platelets (*A*) and their membranes (*B*). ADP was added to the final concentration of 6 µM/10^8 cells or 0.15 µM/200 µg of protein membrane. Fg: 0.01 µM/200 µg of protein membrane

shown in Figure 1. From these spectra the excimer-to-monomer intensity ratio was determined both for the control and the ADP-treated platelets. Data presented in Fig. 2*A* show a decrease in the excimer/monomer ratio indicative of a reduction in the rate of lateral diffusion of pyrene in the membranes of the ADP-treated platelets (decrease in membrane fluidity). A similar effect of ADP on the membrane fluidity was observed when intact platelets were replaced by the isolated membranes (Fig. 2*B*). A decrease of membrane fluidity was even more pronounced in the presence of fibrinogen. In the case of the intact platelets the effect of fibrinogen on the membrane fluidity was not studied due to the strong interference of the additive fibrinogen (0.2 mg/ml) at the excitation and fluorescence bands of pyrene.

Table. Fluorescence anisotropy r of fluorescent probes bound to the control platelet membranes and to the platelet membranes in the presence of ADP and fibrinogen

	Anisotropy r		
	control	+ADP	+Fg
DPH	0.176±0.005	0.181±0.012	0.196±0.002[b]
ANS	0.154±0.002	0.176±0.012[a]	0.180±0.007[b]
Fluorescamine	0.118±0.012	0.127±0.010	0.131±0.011[a]
3-PM	0.087±0.006	0.086±0.006	0.077±0.005[b]
Tryptophan	0.138±0.005	0.138±0.005	0.120±0.005[b]

[a] $P<0.05$.
[b] $P<0.01$ (one-tailed Student's "t" test).

DPH Fluorescence Anisotropy

DPH is another fluorescent probe which is known to be an indicator of changes in the fluidity of the membrane lipid core (Bouchy, Donner & André, 1981; Steiner, 1981). Steady-state fluorescence studies using this probe showed an increased anisotropy in the fibrinogen-treated platelet membranes when compared to the control, indicating increased membrane lipid microviscosity (Table).

I(12,3)-Labeled Platelets

Additional confirmation of ADP and fibrinogen-induced decrease in membrane fluidity comes from spin label studies employing a fatty acid analog I(12,3). Evidence was presented (Sauerheber et al., 1980) which indicated that spectra of this probe principally reflect properties of the platelet surface membrane lipids. An ESR spectrum of washed human platelets labeled with the I(12,3) spin probe is shown in Fig. 3. The order parameter calculated from such a spectrum was increased in the ADP-activated platelets when compared to the intact platelets. Fibrinogen binding to the ADP-activated platelets resulted in a further increase of the order parameter (Fig. 4), indicating elevated rigidity of the membrane lipids.

Such an increased membrane rigidity usually results in a greater exposure of membrane proteins (Shinitzky & Inbar, 1976). Therefore, this effect may be important for the ADP and fibrinogen-treated platelets.

ANS Steady-State Emission Spectra

In view of its amphiphilic nature, ANS is known to be a specific probe for apolar-polar interfaces. Such interfaces could be due to phospholipids or hydrophobic regions in membrane proteins. The

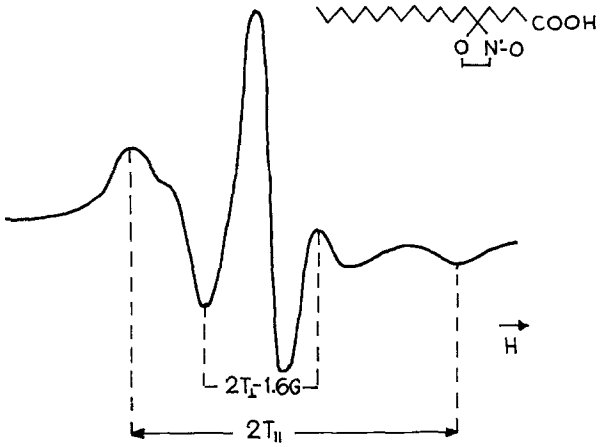

Fig. 3. ESR spectrum of I(12,3)-labeled human blood platelets. $T_{\parallel}$ and $T_{\perp}$ were measured as indicated. The probe-to-cell ratio was 10 μg per 5×10^8 pl. Experimental details are given in the text

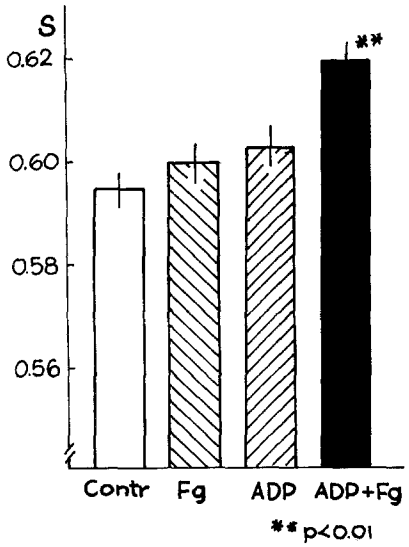

Fig. 4. Effect of ADP (6 μM per 10^8 pl), Fg (0.2 mg per 10^8 pl) and both factors together on the platelet order parameter S of I(12,3) label. Values were calculated as described in the text

binding of ANS with the human platelet membranes was analyzed in the system containing samples of membrane suspension corresponding to 150 μg of protein per ml, and increasing concentrations of ANS. The obtained emission spectra of the membrane-bound ANS are presented in Fig. 5. There was only a slight shift of the emission maximum, from 476 to 480 nm, accompanied by the enlargement of the fluorescence intensity when the ANS concentration was increased from 10 to 50 μM. It is noteworthy that neither ADP nor fibrinogen influenced significantly the fluorescence intensity of the membrane-bound ANS. Similary, when the washed human platelets were used instead of the isolated platelet membranes, ADP

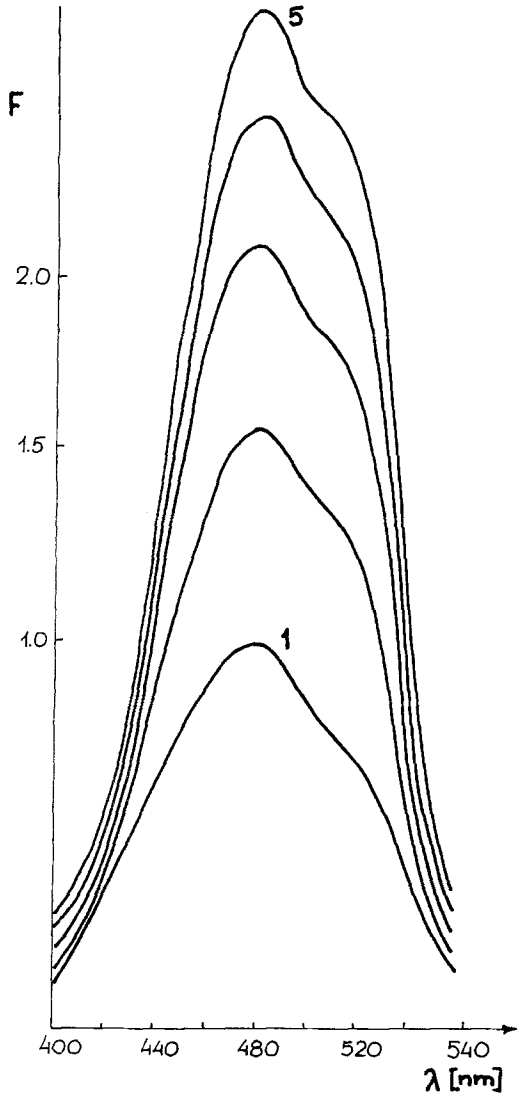

Fig. 5. Fluorescence emission spectra of ANS bound to the platelet membranes at the constant membrane concentration (150 μg of protein per ml). $\lambda_{ex}=370$ nm. ANS concentration varied from 10 to 50 μM

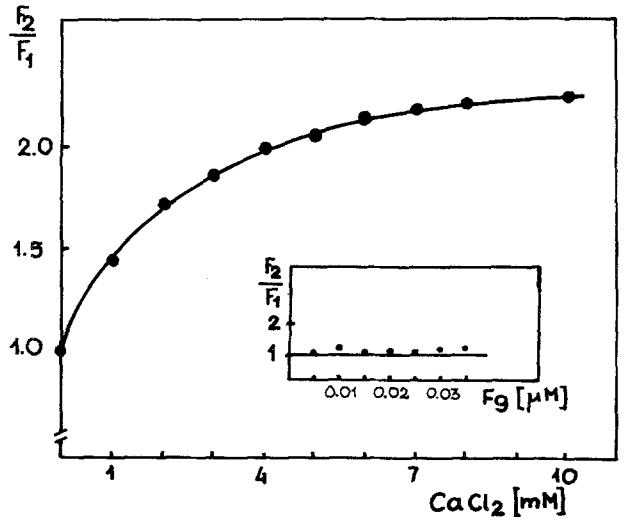

Fig. 6. Effect of $CaCl_2$ on the enhancement of ANS fluorescence in the platelet membranes. Figure inset: Effect of fibrinogen on the ANS fluoresence. $\lambda_{ex}=370$ nm, $\lambda_{em}=478$ nm

and fibrinogen did not induce any detectable changes in the fluorescence intensity of the membrane-associated ANS (Fig. 6, inset). The latter experiments were performed in the absence of calcium ions to avoid platelet aggregation (Marguerie et al., 1980).

The fluorescence intensity of ANS bound to the platelet membranes was enhanced in the presence of calcium ions and reached its maximum at concentration of 7 mM (Fig. 6). It should be pointed out, that in contrast to other plasma proteins, fibrinogen alone failed to bind the ANS molecules.

Figure 7 shows the emission spectra of the platelet membranes in the absence (curve 1) and

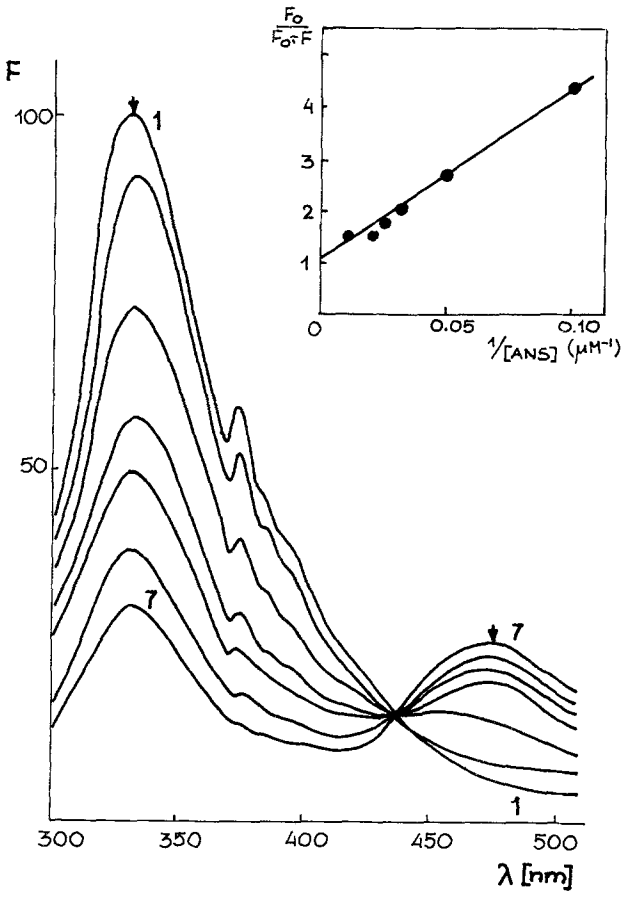

Fig. 7. Tryptophan fluorescence spectra of the platelet membranes in the absence (curve 1) and presence (curves 2–7) of 10, 20, 30, 40, 60 and 80 μM of ANS (membrane concentration 200 μg of protein per ml). $\lambda_{ex}=295$ nm. Figure inset: double reciprocal plot of fluorescence intensity of tryptophan *vs.* ANS concentration. F_0 and F denote the tryptophan fluorescence in the absence and presence of ANS, respectively

in the presence (curves 2–7) of increasing amounts of ANS. In the absence of ANS, fluorescence is emitted by tryptophan residues with maximum at 333 nm/λ_{em} = 295 nm). While adding the increasing amounts of ANS the tryptophyl fluorescence band is quenched and a second peak appears with a maximum at 476 nm. Since free ANS does not fluoresce under these conditions the new band represents tryptophan-excited ANS fluorescence. The inset of Fig. 7 shows the double-reciprocal plot of the decrease in the fluorescence intensity of the tryptophan *vs.* ANS concentration. The intercept of the ordinate gives the reciprocal maximum transfer efficiency corresponding to a state of complete occupation of all ANS binding sites (Andley & Chakrabarti, 1981). From such a plot the maximal transfer efficiency was calculated to be 0.95. There was again no effect of either ADP or fibrinogen on the energy transfer from tryptophans to the ANS attached to platelet membranes, indicating no alterations in the membrane localization of ANS by the additives.

ANS Fluorescence Anisotropy

Significant changes in the fluorescence anisotropy of ANS bound to platelet membranes were observed in the presence of ADP and fibrinogen (Table). This may indicate a decreased rotational mobility of ANS moiety at the polar-nonpolar interface of the fibrinogen complexed with the platelet membranes.

Fluorescamine Fluorescence Anisotropy

Fluorescamine covalently binds to the primary amines of the cell surface, including membrane proteins, glycoproteins, and phospholipids such as phosphatidylethanolamine and phosphatidylserine (Udenfriend et al., 1972; Hawkes, Meehan & Bissell, 1976). As shown in the Table, the early course of fibrinogen binding is associated with slightly increased anisotropy. These changes were insignificant and smaller than those of DPH or ANS.

N-(3-Pyrene) Maleimide Anisotropy

This probe is fluorescent when bound to an −SH group of proteins but not in aqueous solutions. It is commonly used in fluorescence polarization studies owing to its long lifetime (Weltman & Sharo, 1973; Wu, Yarbrough & Wu, 1976). Upon fibrinogen addition to the platelet membranes the fluorescence anisotropy of membrane-bound 3-PM indicated an increased rotational mobility as compared to the control (Table).

Tryptophan Fluorescence

Tryptophan fluorescence of the membrane components might be also used to approach dynamic properties of the membrane proteins. There was no alteration of the fluorescence intensity of tryptophan residues in the membrane complexed with fibrinogen. Additive fibrinogen (0.01 μM) did not influence significantly the tryptophan fluorescence of membrane proteins. However, their fluorescence anisotropy was reduced due to an increased fluctuational mobility of the membrane proteins and/or their conformational changes (Table).

Discussion

Fluorescence probes provide a sensitive tool for the evaluation of dynamic properties of biological membranes. This technique was applied in our study to monitor changes in microviscosity of the platelet membranes associated with fibrinogen binding. As fluoresent probes we used DPH and pyrene to monitor the changes in the lipid core of platelet membranes, ANS a specific probe for polar-nonpolar interfaces, and pyrene maleimide and fluorescamine attached to the platelet membrane proteins. In previous studies they were reported to be highly efficient in measuring microviscosities of cell membranes. In Fig. 8 membrane localization of the fluorescent probes used in these studies is described. The microviscosity changes caused by fibrinogen binding appear to be quite significant and are detectable in different regions of the platelet membranes.

In addition, order parameter which measures the fluidity of the lipid environment of the incorporated probe may be calculated from the electron spin resonance (ESR) spectra of 5-nitroxide sterate I(12,3)-labeled platelets. The I(12,3)-label probably intercalate into the platelet membrane so that the carboxyl group is relatively anchored to the polar surface, while the more mobile methyl terminus lies within the interior of the membrane (Fig. 8).

When fibrinogen molecules are bound to the platelet membranes, both the enlarged fluorescence anisotropy of DPH and reduced rate of the lateral diffusion of pyrene indicate a decrease in the platelet membrane fluidity. The increase of the order parameter of the membrane lipids confirms the above investigation. This observation is in agreement with the findings of the hindered rotational freedom of DPH indicating a decreased fluidity of membrane lipids in the platelets activated with thrombin (Nathan et al., 1979, 1980).

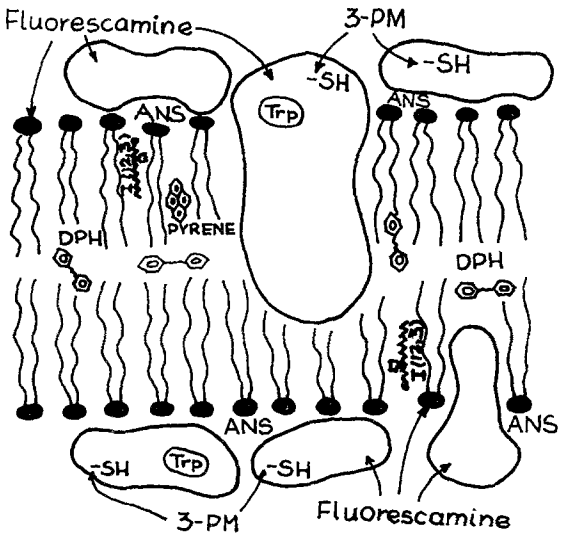

Fig. 8. Schematic representation of platelet membrane with lipid bilayers and cell surface membrane proteins. Fluorescent probes: pyrene, 1,6-diphenyl-1,3,5-hexatriene (DPH), 1-anilino-8-naphthalene sulfonate (ANS), N-3-pyrene maleimide (3-PM), and fluorescamine monitor specifc areas of the membrane. Spin probe I(12,3) is located in hydrocarbon core. Besides, tryptophan (Trp) fluorescence of membrane proteins represents a parameter related to their dynamic properties

Fluidity of the platelet membranes may be influenced by the following structural modifications: a) changes in the ratio of cholesterol to phospholipids, sphingomyelin to lecithin, and unsaturated to saturated fatty acids; b) altered lipid-protein interactions. Under the conditions used changes in the lipid composition of the cell membrane are rather unlikely to occur. It seems that alterations in the lipid-protein interactions associated with binding of fibrinogen to fibrinogen receptors are responsible for the deviations in lipid rigidity of the platelet membranes. On the other hand modification of lipid fluidity is a generalized membrane phenomenon which itself may modulate functional properties of membrane proteins and can be an important intrinsic event in the process of platelet aggregation. It has been shown that an increased membrane lipid rigidity leads to a greater exposure of membrane proteins, including fibrinogen receptors. This might facilitate a formation of molecular linkages between neighboring platelets. A dimeric fibrinogen perfectly fits to this model as a bivalent molecule.

In addition to the changes of the mobility of the probes incorporated into the lipid layer, the binding of fibrinogen to the platelet membranes caused an increase of mobility of fluorescent probe linked to membrane -SH groups. It does suggest that binding of fibrinogen molecules with platelet receptors is associated with the increased mobility of the platelet membrane proteins. This was further supported by an analysis of the platelet membrane tryptophan residues.

On the other hand our spectrofluorimetric studies showed that platelet membrane vesicles efficiently bind ANS. The fluorescence yield and the emission maximum prove that the microenvironment of the ANS molecules attached to the membrane is much less polar than that of the hydrated surfaces. Insignificant displacement of the fluorescence maximum associated with the augmented binding of ANS to the membranes suggests that the probe is in the membrane areas showing nearly the same polarity. The energy transfer measurements allow to locate the ANS molecules in contact with membrane proteins or very close to them. Neither ADP nor fibrinogen affected the yield of the energy transfer from membrane protein tryptophan residues to ANS.

Under condition of ANS excess, being in equilibrium with the membranes, the main factor affecting the ANS fluorescence intensity is the surface charge of the membrane determining the amount of ANS bound to the membranes. There was also no alteration of the fluorescence intensity of ANS bound to the platelet membranes in the presence of ADP or fibrinogen. This was observed both for the intact platelets and their isolated membranes. It means that the binding of fibrinogen molecules does not change the surface charge of the platelet membranes, and thus, it does not increase the fluorescence efficiency of ANS.

In contrast to fibrinogen, calcium ions caused an increase of fluorescence intensity resulting from the more efficient binding of ANS to the platelet membranes. Calcium ions complex with the negatively charged regions of the membrane phospholipid molecules and partially neutralized them. It explains the enlarged fluorescence yield of ANS.

The fluorescence polarization studies showed that fibrinogen and ADP significantly reduce the rotational mobility of the probe attached to the platelet membranes. The latter probably results from the shielding of the ANS molecules by fibrinogen associated with the membrane receptors or by the membrane proteins alone.

In summary, our fluorescence and spin label probes analyses showed that the reaction of fibrinogen molecules with the membrane receptors is accompanied by an increase of the membrane lipid rigidity, which seems to be indirect in nature and is mediated by altered protein-lipid interactions, and augmented mobility of the membrane protein components.

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