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MEMBRANE DYNAMIC ALTERATIONS ASSOCIATED WITH ACTIVATION OF HUMAN PLATELETS BY THROMBIN

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Summary

Two fluorescent probes, *N*-carboxymethylisatoic anhydride, which binds to membrane proteins, and 1,6-diphenyl-1,3,5-hexatriene, a lipophilic label, have been used to follow membrane microenvironmental changes. Activation of human platelets by thrombin resulted in a simultaneous increase in values of fluorescence polarization (*P*) of both probes during the stages of shape change and secretion, which further increased during platelet aggregation. The similar pattern of changes in *P* for both probes indicates the interdependence of lipids and proteins in the activated platelet membrane.

Thrombin is one of the most potent activators of blood platelets. Upon activation, platelets undergo dramatic metabolic and morphologic modifications, expressed in shape change, secretion and aggregation. The membrane changes related to thrombin-induced platelet activation are not clear [1]. Fluorescent probes which bind selectively to membrane constituents could assist in following membrane changes. Indeed, fluorescence polarization studies were applied effectively to probe membrane changes in platelets enriched with cholesterol and in complement-mediated lysis of platelets [2—4].

Using a lipophilic fluorescent probe we have shown that platelet activation by thrombin is accompanied by a decrease in rotational mobility of membrane

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Abbreviations: SDS, sodium dodecyl sulfate; *P*, value of fluorescence polarization.

lipids [5]. In the present study we report that platelet membrane proteins exhibit similar and simultaneous dynamic changes.

Two fluorescent probes have been employed: the polar *N*-carboxymethylisatoic anhydride which binds covalently to membrane proteins containing free-NH_2 group [6,7], and 1,6-diphenyl-1,3,5-hexatriene, a lipophilic non-covalent label [8—11]. Fluorescence excitation and emission characteristics of these two probes can be followed without interference from the inherent fluorescence of platelet proteins.

Human blood platelets were isolated as described [12]. Platelets, $1 \cdot 10^9$/ml and $1.5 \cdot 10^8$/ml, were incubated in medium A for 1 h at 37° C with 0.2 mg/ml of *N*-carboxymethylisatoic anhydride and at 25° C with $1 \cdot 10^{-6}$ M 1,6-diphenyl-1,3,5-hexatriene [2], respectively. Excess reagent was washed off (by two cycles and one cycle of centrifugation for *N*-carboxymethylisatoic anhydride- and 1,6-diphenyl-1,3,5-hexatriene-labeled platelets, respectively) and the platelets were finally suspended in Tyrode's buffer fortified with 0.1 mg/ml apyrase. The labeling of platelets caused no measurable damage to the platelets, as evaluated by criteria of intactness and function. Thus, no excess leakage of lactate dehydrogenase, a cytoplasmic enzyme and of [^{14}C]serotonin, a granular marker, from the labeled platelets took place and platelet activation with thrombin proceeded normally.

The polar *N*-carboxymethylisatoic anhydride did not penetrate intact platelets. Thus, only two to three fluorescent bands were revealed by SDS-polyacrylamide gel electrophoresis of intact *N*-carboxymethylisatoic anhydride-labeled platelets. These bands corresponded to glycoprotein bands, stained with periodate-fuchsin. In contrast, labeling of lysed platelets with *N*-carboxymethylisatoic anhydride revealed numerous fluorescent bands. No labeling of amino phospholipids took place in *N*-carboxymethylisatoic anhydride-labeled platelets under the conditions described. Thus, phospholipid extracts [5] from *N*-carboxymethylisatoic anhydride-labeled platelets and from unlabeled platelets with equal phosphate content [13] showed identical emission spectra.

Fluorescence measurements were carried out in a modified MPF-44 Perkin Elmer spectrofluorimeter, equipped with a device for stirring at 1100 rev./min at 37° C. Fluorescence polarization (*P*) was monitored either by fast flipping of the emission polarizer or by running two equivalent samples with the polarizers set at parallel orientation for one sample and perpendicular for the other. The fluorescence values were corrected for the scatter contribution of unlabeled platelets (approx. 16 and 2% for *N*-carboxymethylisatoic anhydride- and 1,6-diphenyl-1,3,5-hexatriene-labeled platelets, respectively). Platelet aggregation was monitored, prior and subsequent to the fluorescence measurements, in the same spectrofluorimeter, but with the excitation and emission monochromators set at 600 nm. The modified spectrofluorimeter and the conventional Bryton aggregometer showed identical aggregation patterns.

The course of aggregation of platelets activated by thrombin and the changes in fluorescence polarization associated with aggregation are shown in Fig. 1. Appearance of bulky aggregates at advanced aggregation stages (from approx. 1—2 min after the addition of thrombin) prevented reproducible results and the studies were therefore limited to the early stages of aggregation. Aggregation was clearly accompanied by an increase in *P* of both *N*-carboxymethylisatoic

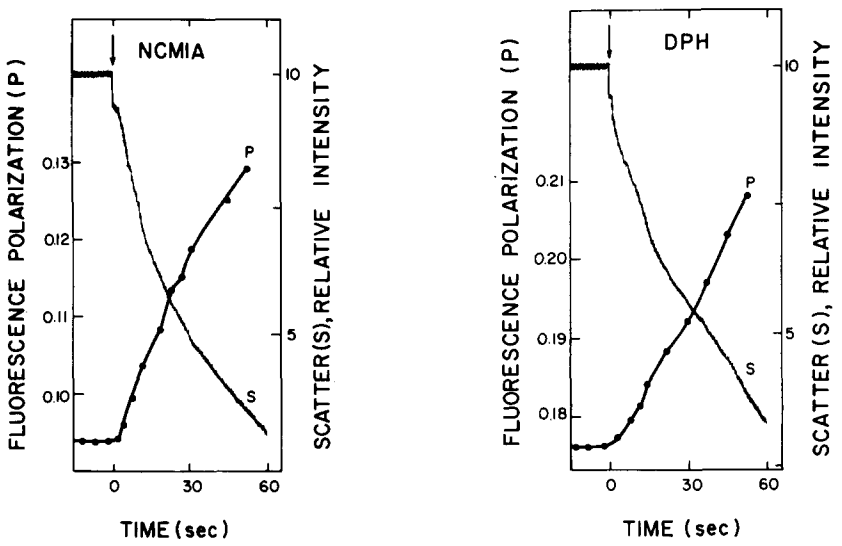

Fig. 1. Rotational dynamics of *N*-carboxymethylisatoic anhydride-labeled and 1,6-diphenyl-1,3,5-hexatriene-labeled platelets during aggregation induced by thrombin. Measurements carried out on 3-ml platelet suspension, $(3 \pm 0.5) \cdot 10^8$ platelets/ml. Aggregation was induced by thrombin (1 unit/ml) added at zero time (arrow). Excitation and emission slits were 4 nm for scatter and 4 and 20 nm for *P* measurements, respectively. With excitation at 345 and 350 nm fluorescence emission was followed at 425 and 430 nm for *N*-carboxymethylisatoic anhydride (NCMIA) and 1,6-diphenyl-1,3,5-hexatriene (DPH), respectively. A 390 nm cut off filter was used. *P* was calculated according to the method of Rosenthal et al. [11] and scatter (*S*) was followed at 600 nm.

anhydride- and 1,6-diphenyl-1,3,5-hexatriene-labeled platelets, representing a decreased rotational freedom of the probes in their respective microenvironment. It is unlikely that the increased polarization values resulted from decreased lifetime of the probes [8—11], since fluorescence intensity under these conditions did not decline [5]. In addition, the increase in *P* values with aggregation, albeit decreased scatter, indicates that the increase is not due to primary scattered radiation.

When activated by thrombin in the presence of EDTA, labeled platelets rapidly changed shape and secreted their intragranular content but did not aggregate, as shown for unlabeled platelets [14]. Fig. 2 shows that under these conditions thrombin caused an increase in *P* in both *N*-carboxymethylisatoic anhydride- and 1,6-diphenyl-1,3,5-hexatriene-labeled platelets. The overall increase in *P* in aggregating platelets reflects two types of alteration: changes corresponding to individual activated platelets (Fig. 2) and changes due to the linkages among these platelets. Apparently, the linkages between the pseudopod membranes further decrease the rotational freedom of the probe molecules. Increased *P* values, despite the proteolysis induced by thrombin, indicate that these increases in *P* result from higher membrane microviscosity. The elevation in membrane lipid microviscosity and the decreased rotational freedom of membrane proteins may reflect an overall increase in lipid packing density and in lipid-lipid interaction, as well as an increased protein-protein interaction [15]. The very similar kinetics of changes in *P* of *N*-carboxymethylisatoic anhydride- and 1,6-diphenyl-1,3,5-hexatriene-labeled systems indicate the inter-

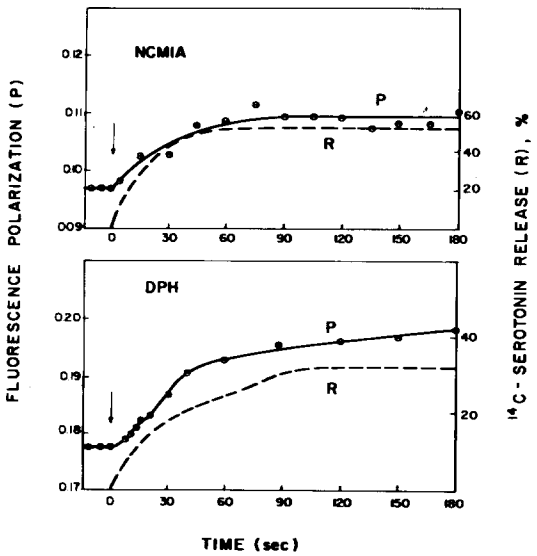

Fig. 2. The effect of thrombin, in the presence of 5 mM EDTA, on the rotational dynamics and on serotonin release of *N*-carboxymethylisatoic anhydride-labeled (NCMIA) and 1,6-diphenyl-1,3,5-hexatriene-labeled (DPH) platelets. Thrombin (1 unit/ml) was added at zero time (arrow). The procedure for *P* measurement detailed in Fig. 1 was followed. Release of [^{14}C]serotonin, measured according to the method of Massini and Lüscher [25], is represented by the dashed curve.

dependence of lipids and proteins in the membrane.

Several functional implications for the elevated microviscosity in activated platelets may be considered. Lipids may play a role in modulating membrane receptors and due to increased lipid-lipid interaction, membrane proteins thus become differently exposed to aqueous surroundings, as suggested for other membranes [16,17]. Indeed, the sensitivity of platelets to aggregation by epinephrine and ADP was enhanced in platelets in which lipid microviscosity was elevated by incorporated cholesterol [2,18]. In addition, membrane phospholipids may become more favorably exposed to endogenous phospholipase A_2, the activity of which was markedly stimulated with increased lipid microviscosity [19].

Uncertainties in steady-state fluorescence measurements have been pointed out [9,10]. These arise particularly from the average nature of measurement, the uncertainty in the 1,6-diphenyl-1,3,5-hexatriene location, and the exact interpretation of such dynamic data, i.e., microviscosity vs. anisotropy effects [10]. The observed depolarization in the nanosecond time-scale could arise from an independent motion of the bound probe and from segmental motion of the labeled protein. Cherry [20] pointed out that rotational correlation times for membrane proteins [21] are much slower than those of proteins in aqueous solution [22]. Both fluorescent lectin [23] and *N*-carboxymethylisatoic anhydride label plasma membrane glycoproteins in respective cell systems, and exhibited a rotational time-scale corresponding to aqueous solution of proteins. Depolarization of fluorescently labeled glycoproteins could arise from the fast rotation of the aqueously exposed portion of the glycoproteins, presumably around the protein axis which is normal to the plane of the membrane [21].

The crucial role played by the plasma membrane in platelet activation, and the parallel responses of the polar, non-penetrating *N*-carboxymethylisatoic anhydride probe and the lipophilic 1,6-diphenyl-1,3,5-hexatriene, do indicate that the increase in *P* values coinciding with platelet activation may be attributed primarily to changes occurring in the plasma membrane of the activated platelet. Clustering of membrane proteins and an otherwise decrease in their mobility may still be controlled by cytoskeleton proteins [24].

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