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Membrane fluidity of non-activated and activated human blood platelets

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The steady-state fluorescence anisotropy of membranes labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH) or its 4′-trimethylammonio derivative, TMA-DPH, is generally considered a measure for the lipid order and, hence, inversely related to membrane fluidity. We now report that anisotropy values of DPH- and TMA-DPH-labeled human platelets are considerably influenced by experimental conditions like the platelet concentration, which do not affect membrane fluidity. Activation of platelets with thrombin increases, but activation with ionomycin decreases anisotropy values with both labels. Such anisotropy changes are not detected in platelet membranes or platelet lipids, when isolated after activation of the intact platelets. We present evidence that the anisotropy changes of intact platelets are not a consequence of modified lipid composition (e.g., as would be induced by phospholipase A_2 activity) but are, at least partially, caused by changed optical properties of the cell suspension. Measurement of membrane fluidity of platelets by fluorescence polarization is severely hindered by a high turbidity of the platelet suspension and also by changes in the turbidity and platelet morphology during the activation process.

Introduction

The activation of blood platelets is initiated by a set of specific extracellular signals. There is firm evidence now that the platelet plasma membrane has a major role in transduction of the activating signal [1,2]. Various authors have discussed a role of (plasma) membrane fluidity in the process of platelet activation. The majority of these studies was done using the technique of steady-state fluorescence polarization with a variety of fluorescent probes [3–10]. As predicted by theory, a high anisotropy (r_s) or polarization (P) value of the emitted fluorescent light is interpreted as indicative for a high degree of order in the probe environment and, thus, a less fluid membrane [11,12]. For instance, the lipophilic probe 1,6-diphenyl-1,3,5-hexatriene (DPH) shows increased r_s values (i.e., reduced membrane fluidity) in platelets and platelet membranes that were artificially enriched in cholesterol [3,13]. The reduced fluidity was correlated with a higher tendency of the platelets to become activated by thrombin or ADP [13,14]. DPH [4,7] and its 4′-trimethylammonio derivative TMA-DPH [8,9] were also used to demonstrate changes in r_s during platelet activation. Stimulation of platelets with thrombin resulted in increased r_s or P values [4], whereas activation with calcium ionophore gave decreased r_s values [7,9]. Similar anisotropy changes have been measured for fluorescent probes with high-binding affinity for membrane proteins, demonstrating a (transient) increase of r_s upon thrombin-induced platelet activation [5,6].

When membrane fluidity was studied with spin labels instead of fluorescent labels, however, different results were obtained. The order parameter in spin-labeled cell membranes was not increased but decreased upon platelet stimulation with thrombin or other agonists [15,16], whereas activation with calcium ionophore A23187 had no effect on membrane rigidity [16]. Thus, depending on the method of analysis (fluorescence polarization or ESR), there is a discrepancy in the type of apparent changes in the physical state of activated platelet membranes.

In the present fluorescent study, steady-state r_s during platelet activation is followed with two probes: DPH, which labels all cellular membranes, and TMA-DPH as a specific probe for the platelet plasma mem-

Abbreviations: DPH, 1,6-diphenylhexa-1,3,5-triene; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; TMA-DPH, 1-[4-(trimethylammonio)phenyl]-6-phenylhexa-1,3,5-triene.

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brane [9]. We report that experimental conditions, especially those modifying the optical density of a platelet suspension, have considerable effects on the fluorescence measurements and we question the evidence for changes in membrane fluidity, as revealed by steady-state fluorescence polarization.

Materials and Methods

Materials. Thrombin was purified from bovine plasma to homogeneity according to Rosing et al. [17]. Ionomycin was purchased from Serva (Heidelberg, F.R.G.), DPH and TMA-DPH were from Molecular Probes (Junction City, OR) and *sn*-1-stearoyl-2-[1-^{14}C]arachidonoylphosphatidylcholine was supplied by Amersham (Amersham, U.K.).

Platelet preparation. Blood (80 ml) was obtained from healthy volunteers who had not taken medication for at least two weeks, and was anticoagulated with 6% (v/v) ACD. Platelets were isolated by differential centrifugation and washed two times, as described by Bevers et al. [18]. The washed platelets were finally suspended in buffer A containing 136 mM NaCl, 10 mM Hepes, 5 mM glucose, 2.7 mM KCl and 2 mM $MgCl_2$ (pH 7.4) (albumin was not included, because of its interference with fluorescence measurements). The number of non-activated platelets in suspension was counted with a Thrombocounter-C from Coulter Electronics (Luton, U.K.) or was calculated from the optical density at 405 nm, using a calibration curve. Total platelet membranes were prepared by sonication for 10 min, with 10 s intervals, using a M.S.E. (Crawley, U.K.) probe sonicator at an amplitude of 9 μm. Vesicles from platelet lipids were made by chloroform extraction of intact platelets and sonication of the isolated lipids, as described before [10].

Platelet activation. Washed platelets ($5 \cdot 10^7$ platelets/ml) were activated in a 3 ml quartz cuvette under moderately stirring (250 rpm) at 37°C in buffer A, in the presence of 1.0 μM TMA-DPH or 0.5 μM DPH.

Anisotropy measurements. Anisotropy of DPH- and TMA-DPH-labeled platelets and lipid extracts was measured by steady-state fluorescence polarization, as described before [10]. Standard conditions of measurement were: $5 \cdot 10^7$ washed platelets/ml (or equivalent of platelet lipids, i.e., 20 μM phospholipids), 0.5 μM DPH (pre-incubated with platelets for 30 min) or 1.0 μM TMA-DPH (no pre-incubation) at 37°C. Platelets were stirred before start of the measurement to facilitate temperature equilibration, but not during data collection. All r_s values given are averaged data (five measurements of three separate samples) and representative for at least three experiments. Background scattering, i.e., the contribution of scattered excitation light to the light intensity measured at the emission wavelength did not exceed 1% of total light intensity, and was neglected.

Various procedures. Platelet phospholipase A_2 (EC 3.1.1.4) activity was determined, using the assays described by Ulevitch et al. [19]. Optical densities were determined spectro-photometrically at 405 nm. Scanning electron microscopy was performed according to Sune and Bienvenue [20] and samples were observed with a Philips (Eindhoven, The Netherlands) CM-12 Electron Microscope at 20 kV.

Results

The steady-state fluorescence anisotropy (r_s) of non-activated human platelets, labeled with either TMA-DPH or DPH, is strongly dependent on the experimental conditions. Stirring of the platelet suspension and higher platelet concentrations both result in considerably decreased r_s values (Table I). A similar concentration dependence of r_s was seen before with rat platelets [10]. Stirring and concentration have much smaller effect on r_s values of vesicles prepared from platelet lipids (Table I). Apparently, the r_s of intact platelets is influenced by experimental factors that are unrelated to membrane fluidity. On the other hand, r_s values of platelets and platelet lipid vesicles respond quite similarly to variation of temperature (Table I), thus indicating that changes in lipid order can nevertheless be detected in the intact cells.

Activation of platelets results in rapid, persistent changes of r_s, which are quite similar for either probe TMA-DPH or DPH. However, the two activators

TABLE I

Effect of experimental conditions on steady-state anisotropy (r_s) of TMA-DPH- and DPH-labeled platelets

Standard conditions were $5 \cdot 10^7$ platelets/ml (or equivalent of platelet lipids), 1.0 μM TMA-DPH or 0.5 μM DPH, 37°C and no stirring. Changes from standard conditions are as indicated. Mean r_s values (S.E. is 0.001–0.002, $n = 3$) are given.

	r_s	
	TMA-DPH	DPH
Platelets		
Stirring rate: 0 → 1200 rpm	0.237 → 0.214	0.173 → 0.164
Platelet concentration:		
$2 \rightarrow 20 \cdot 10^7$ platelets/ml	0.249 → 0.188	0.184 → 0.137
Temperature: 20 → 37°C	0.260 → 0.237	0.199 → 0.173
Platelet activation: 0 → 1 min		
thrombin (2 nM)	0.238 → 0.258	0.173 → 0.186
ionomycin (0.5 mM)		
+ $CaCl_2$ (1 mM)	0.238 → 0.217	0.173 → 0.159
Platelet lipids		
Stirring rate: 0 → 1200 rpm	0.234 → 0.234	0.168 → 0.169
Platelet lipid concentration:		
$2 \rightarrow 20 \cdot 10^7$ platelets/ml	0.234 → 0.224	0.168 → 0.172
Temperature: 20 → 37°C	0.256 → 0.233	0.210 → 0.169

TABLE II

Effect of platelet activation on TMA-DPH anisotropy in various platelet preparations

Platelets were activated for 2 min as indicated, and used for preparation of platelet membranes and platelet lipids. Anisotropy measurements were at $5 \cdot 10^7$ platelets/ml or equivalent amounts of platelet membranes or lipids (20 μM phospholipid). Data are mean values ± S.E. ($n = 3$).

Activator	r_s		
	platelets	platelet membranes	platelet lipids
None	0.242 ± 0.001	0.242 ± 0.002 [a]	0.234 ± 0.002 [a]
Thrombin (2 nM)	0.258 ± 0.001	0.242 ± 0.002	0.233 ± 0.001
+ EDTA (2 mM)	0.250 ± 0.002	n.d. [b]	n.d. [b]
Ionomycin (0.5 μM)			
+ $CaCl_2$ (1 mM)	0.216 ± 0.002	0.242 ± 0.002	0.237 ± 0.001
+ EGTA (2 mM)	0.246 ± 0.002	0.243 ± 0.001	0.238 ± 0.001

[a] Addition of thrombin or ionomycin did not influence r_s value.
[b] n.d., not determined.

thrombin and ionomycin induce a different type of change: thrombin results in higher, but ionomycin in combination with calcium gives lower r_s values (Table I). Similar to thrombin, stimulation of platelets with ADP, concanavalin A or vasopressin results in increased r_s values (data not shown). The thrombin effect can be suppressed partially by EDTA (Table II). The ionomycin-induced decrease in r_s requires extracellular calcium, since in the presence of EGTA r_s is slightly increased (Table II).

The r_s of unstimulated platelets is far more dependent on platelet concentration than that of thrombin- or ionomycin-activated platelets (Fig. 1). Anisotropy values of platelet membranes (Fig. 1) and of platelet lipids (Table I) both are relatively concentration-independent. Thus, a high concentration effect is typical for the TMA-DPH and DPH r_s of non-activated, intact platelets.

The question arises, whether the anisotropy changes upon platelet activation, in fact, are a consequence of altered membrane fluidity, i.e., a changed ordening degree of the membrane components. In a first approach to answer this question, we compared r_s values of platelets with those of platelet membrane and lipid vesicles, isolated before and after activation of the intact cells. Table II shows that the r_s of membranes and lipids, in contrast to that of intact platelets, is not affected by the activation process. Only after ionomycin activation, r_s of the lipid vesicles is slightly increased compared to that of lipids from unstimulated platelets, possibly as a consequence of phospholipase A_2 activity (see below).

Quantitatively important changes in lipid composition during platelet activation are effected by platelet phospholipase A_2 [21]. In model membranes, action of phospholipase A_2 resulted in an increased lipid order and, thus, in rigidification [22]. This is confirmed in platelet membranes, where action of endogenous phospholipase A_2 is accompanied by increase of r_s (Table III). Taken together, the almost unchanged anisotropy in membranes and lipids prepared from activated platelets makes it unlikely that r_s changes of intact stimulated cells are a consequence of modified lipid composition.

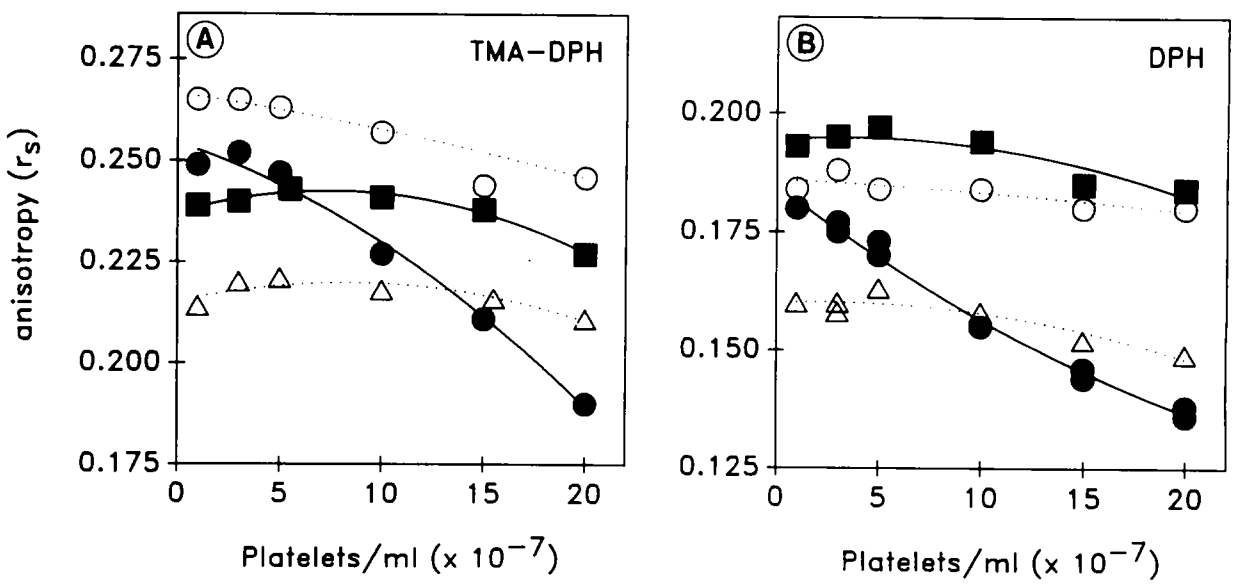

Fig. 1. Effect of platelet concentration on steady-state anisotropy (r_s) values. Platelets were labeled with 1 μM TMA-DPH (A) or with 0.5 μM DPH (B). Shown are r_s values of non-activated platelets (●), of platelets activated for 1 min with 2 nM thrombin (○) or with 0.5 μM ionomycin + 1 mM $CaCl_2$ (△). Additionally, r_s values are shown of platelet membranes, made by sonication of non-activated platelets (■). Data are given of one (A) or two (B) representative experiments. Optical densities (405 nm) at a concentration of $5 \cdot 10^7$ platelets/ml were: 0.73, 0.60, 0.33 and 0.19 for non-activated platelets, for thrombin- and ionomycin-activated platelets and platelet membranes, respectively.

Another factor, known to affect fluorescence anisotropy values, is turbidity [23,24]. Platelet suspensions have a very high turbidity. For instance, at a concentration of $5 \cdot 10^7$ platelets/ml, optical density at 405 nm is 0.73. This optical density is considerably decreased upon platelet activation (see legend to Fig. 1). The thrombin-induced decrease in optical density probably is a consequence of formation of platelet aggregates, but ionomycin/$CaCl_2$-induced decrease under our conditions seems not to be caused by aggregates, since single-platelet count was not affected by ionomycin (data not shown, see Ref. 7). The latter observation was confirmed by scanning electron micrographs, which characterize ionomycin/$CaCl_2$-activated platelets as single cells (Fig. 2C). Electron microscopy further shows the typical discoid morphology of non-activated platelets, the loss of discoid shape and pseudopod formation after activation with thrombin or ionomycin/EGTA and the formation of multi-cellular aggregates by thrombin. Platelets activated with ionomycin/$CaCl_2$ are very typical in a spheroid morphology and contain numerous bleb-like structures (Fig. 2).

Discussion

The contrasting effects of thrombin and ionomycin/$CaCl_2$ on platelet r_s, as found with either probe TMA-DPH and DPH, are in agreement with the results of various authors, who generally measured r_s changes with only a limited set of activators using one fluorescent probe [4–7,9]. We now show that the magnitude of the r_s change is strongly dependent on platelet concentration: the thrombin-induced increase of r_s, in fact, tends to be zero at low platelet concentrations (Fig. 1). The high turbidity of platelet suspensions seems to be relevant, since anisotropy values are influenced by the optical density and by the scattering of fluorescent light [23,24] and since the optical density of suspended

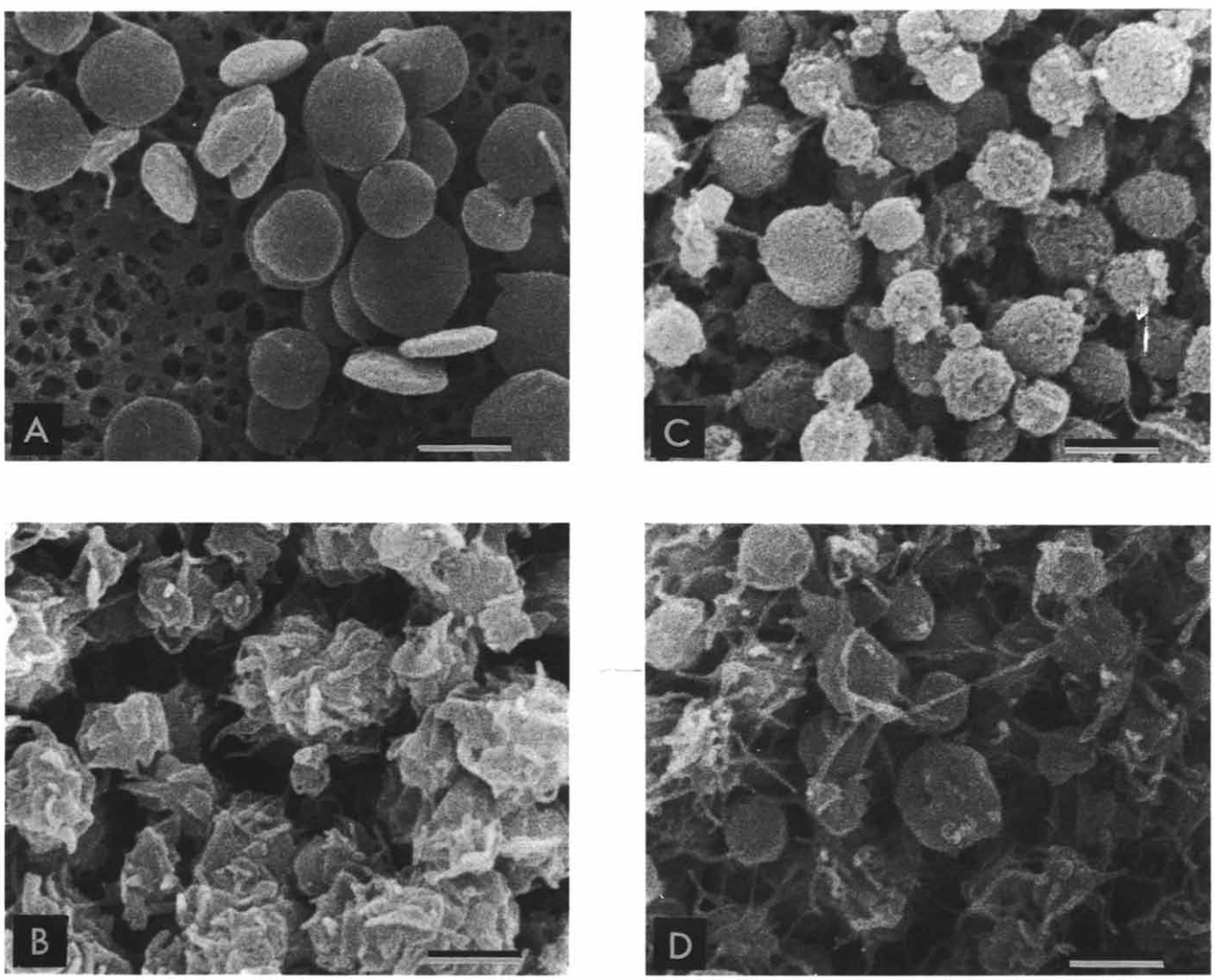

Fig. 2. Scanning electron micrographs of (A) non-activated platelets and of platelets activated with (B) 2 nM thrombin, (C) 0.5 μM ionomycin + 1 mM $CaCl_2$ or with (D) 0.5 μM ionomycin + 1 mM EGTA. Bars indicate 2 μm.

TABLE III

Phospholipase A_2 activity in platelet membranes is associated with increase of r_s

Platelets ($5 \cdot 10^7$/ml) were sonicated with *sn*-1-stearoyl-2-[1-^{14}C]arachidonoylphosphatidylcholine (2.75 nmol, 2.15 GBq/mmol). Then, membranes were incubated with $CaCl_2$ or EGTA (as indicated) at 37°C for 90 min. Samples were taken for measuring TMA-DPH r_s, and lipids were extracted to determine activity of endogenous phospholipase A_2. Data are mean values ± S.E. ($n = 3$).

Incubation	r_s		Phospholipase A_2 activity (% of label)
	platelet membranes	platelet lipids	
None	0.240 ± 0.001	0.234 ± 0.002	1.2
$CaCl_2$ (2 mM)	0.247 ± 0.001	0.239 ± 0.001	4.8
EGTA (2 mM)	0.241 ± 0.001	0.233 ± 0.002	2.1

platelets is strongly affected by platelet concentration and platelet activation.

Generally, scattering of incident or emitted fluorescent light results in decrease of the measured r_s values [24]. Thus, high light-scattering explains the lower anisotropy of more concentrated suspensions of non-activated platelets. On theoretical and experimental grounds, formation of pseudopods and aggregation of platelets are both believed to decrease light-scattering, whereas sphering of the platelets results in increased light-scattering [25]. Platelet activators giving rise to increased anisotropy values are those causing aggregation (e.g., ADP, concanavalin A, thrombin, vasopressin), but also those which induce pseudopod formation in the absence of aggregation (thrombin in presence of EDTA, ionomycin with EGTA). On the other hand, a decreased r_s is seen with activators (ionomycin and A23187 [7] in combination with extracellular calcium), which do not lead to platelet aggregation or pseudopod formation but, instead, generate spheroid single cells with irregular structures (Fig. 2C). Taken together, at least part of the anisotropy changes induced by thrombin and ionomycin can be explained by decrease and increase in light-scattering of the platelet suspension, respectively. Nevertheless, although ionomycin/$CaCl_2$-activated platelets are supposed to be highly scattering because of their spheroid shape [25], they have a very low optical density. The reason for this apparent discrepancy is not known. However, an indication that artificial (optical) factors indeed influence fluorescence values of those platelets is the lack of any change in lipid order (i.e., membrane fluidity), when spin-labeled platelets were stimulated with calcium ionophore [16].

Several membrane processes in activated platelets may influence the order of lipids, and thus the overall fluidity of the (plasma) membrane: change of lipid composition, lateral or transversal rearrangements of membrane components, formation of specific lipid domains, fusion of internal membranes with the plasma membrane, or modified membrane–cytoskeleton interaction. We found no evidence for fluidity changes by alterations in platelet lipid composition. Other processes may require the integrity of whole cells, and then consequently cannot be detected in sonicated platelet membranes. However, if lipid order indeed changes in intact activated platelets, this should be detected by various independent methods. In this respect, evidence for modified fluidity, only based on anisotropy measurements is not convincing, since this cannot be confirmed by ESR [15,16].

As a conclusion, the present experiments indicate that measurement of platelet membrane fluidity by fluorescence polarization is severely hindered by the optical properties of the platelet suspension. Since platelet activation is accompanied by considerable changes in optical density and light-scattering, it is difficult to draw conclusions on changes in membrane fluidity from altered fluorescence anisotropy values alone.

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