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Transbilayer incorporation of 1-pyrenebutyltrimethylammonium by blood platelets and its application for analyzing changes in physico-chemical properties of the membrane lipid bilayer induced by platelet activation

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The binding of cationic butyltrimethylammonium derivative of pyrene to bovine platelets was initially rapid and then increased gradually, unlike the bindings of other anionic and neutral derivatives of pyrene tested. The rate of increase in binding of the cationic probe depended on temperature and was due to its incorporation into the cytoplasmic side of the platelet membranes, as shown quantitatively by monitoring decrease in its extractability with albumin. The penetration into the inner membrane compartment did not reach equilibrium even after 4 h at 37°C. Slow penetration of a fluorescent probe such as this is useful in studies on the physico-chemical properties of the outer layer and cytoplasmic side of the platelet membranes and their changes. Initial rapid binding of the cationic probe to platelets, representing the binding of the probe to the outer layer of the plasma membrane, was increased by ionomycin-induced platelet activation. Fluorescence spectra in the presence of a relatively high concentration of the cationic probe showed increase of the excimer of the cationic probe accompanied with the incorporation of the probe to the cytoplasmic side. On ionomycin-induced activation, the excimer-to-monomer intensity ratio of the probe in the cytoplasmic side of the platelet membranes decreased, possibly due to decrease in fluidity of the lipid layer near the probe or change in distribution of the probe.

Introduction

Many fluorescent probes have been used to evaluate the fluidity of lipid bilayers in biological membranes [1]. These fluorophores become localized at different depths in the lipid bilayer depending on their chemical structures and give information on the lipid mobility in the region in which they are located [2–4]. Moreover, due to the asymmetric distribution of lipids in biological membranes [5,6], charged probes also seem to become distributed asymmetrically dependent on their electrical charges. We recently determined the locations of diphenylhexatriene (DPH) and its cationic and anionic derivatives in platelet membranes [7]. Transbilayer movement of the cationic trimethylammonium derivative of DPH (TMA-DPH) was first shown by Bevers et al. [8]. It first binds to the outer layer of the plasma membrane and then gradually penetrates to the cytoplasmic side by a flip process, binding to the cytoplasmic surface of the membrane which has a negative surface potential. Due to this characteristic behavior, TMA-DPH reveals the fluidity of the cytoplasmic side of the platelet membrane as well as that of the outer layer of the plasma membrane [7]. Thus, we demonstrated asymmetry of membrane fluidity in the lipid bilayer of blood platelets by the change in distribution of TMA-DPH from its initial location in the outside layer to its location on the cytoplasmic side at equilibrium [7].

Pyrene derivatives, which are also probes for monitoring membrane fluidity, give information on membrane fluidity by a different mechanism from DPH derivatives. The excimer of pyrene is formed by collision of monomers, which is controlled by the diffusion rate of the latter [9,10]. The excimer-to-monomer ratio of pyrene derivatives has been regarded as a measure of the resistance of solvent against lateral diffusion of the monomer [9,10], and has been used as an index to evaluate the fluidity of the lipid bilayer in biological membranes [10–13]. Like TMA-DPH, the quaternary ammonium 1-pyrenebutyltrimethylammonium derivative is thought to be a promising probe to reveal the asymmetrical membrane fluidity in biological mem-

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branes, but its binding and transport behaviors in biological membranes have not been studied. Therefore, in this work we examined the binding of this cationic derivative of pyrene to whole platelets and investigated its incorporation into the inner membrane compartment in comparison with those of other pyrene derivatives. We also examined the fluorescence spectrum due to excimer formation and its relation with the localization of the cationic probe. To determine the value of this cationic probe for use in studies on changes of the physico-chemical properties of either the outside layer of the plasma membrane or the cytoplasmic side of the platelet membranes, we also examined the effect of platelet activation by ionomycin on the binding of the cationic probe to the outside layer of the plasma membrane and the fluorescent spectrum of the incorporated probe on the cytoplasmic side.

Materials and Methods

Materials

Pyrene derivatives were purchased from Molecular Probe (Eugene, OR). Bovine serum albumin (essentially fatty acid free) was from Sigma Chemicals (St. Louis, MO). Ionomycin was from Calbiochem (LaJolla, CA). Other reagents were all from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of platelet suspension

Platelet-rich plasma was obtained from fresh bovine (Holstein) blood with 10% by volume of ACD anticoagulant solution (74.8 mM sodium citrate, 38.1 mM citric acid and 122 mM dextrose) [14]. The platelet-rich plasma was then centrifuged at $1000 \times g$ for 8 min and the platelets were suspended in Na,K-Tris medium (137 mM NaCl, 5.4 mM KCl, 11 mM dextrose, 25 mM Tris-HCl adjusted to pH 7.4). Spontaneous platelet aggregation during preservation was prevented by adding 129 mM citrate (adjusted to pH 7.4) to this suspension at a volume ratio of 1:9.

Measurement of binding of pyrene derivatives to platelets

The platelet suspension described above at a final concentration of about $8 \cdot 10^5$ platelets/μl was incubated with 1.5 μM concentrations of pyrene derivatives. After incubation for various times, 0.5 ml of the suspension was collected and centrifuged at $3000 \times g$ for 1 min, and 0.2 ml samples of the supernatant and pellet were collected for solubilization with 0.5% sodium dodecyl sulfate (SDS). Fluorescence intensities were measured in a 4010 spectrofluorometer (Hitachi Seisakusho, Tokyo, Japan). The excitation and emission wavelengths used with pyrene derivatives were 342 and 377 nm, respectively. Amounts of binding to platelets were evaluated from both decrease in the concentration of the probe in the supernatant and its increase in the pellet. The resulting values were consistent with each other and mean values from the two evaluations were used.

Measurement of transbilayer incorporation of butyltrimethylammonium derivative

Incorporation of cationic probe into the inner membrane compartment was quantitated by following decrease in extractability with albumin by a modification of a reported method [6]. After incubation of the platelet suspension ($8 \cdot 10^5/\mu$l) with 1.5 μM 1-pyrenebutyltrimethylammonium bromide for various times, 0.5 ml of the suspension was collected and transferred to 1 ml of 3% albumin solution. Albumin is necessary to extract the probes located in the outer leaflet of the plasma membrane because of the high affinity of the cationic probe to the membrane. The samples were centrifuged at $3000 \times g$ for 1 min. The precipitated platelets were washed once more with 1.5 ml of 1% albumin solution, and then solubilized with 0.5% SDS containing Na,K-Tris. The fluorescence intensity of the cationic probe was measured as described above, and the amounts of the probes incorporated into the cytoplasmic side were calculated.

Measurement of excimer formation by the butyltrimethylammonium derivative in platelets

The fluorescence emission spectrum of the cationic probe in the platelet suspension described above was measured after incubation in the presence of 80 μM probe at 37°C with excitation at 342 nm. The fluorescence spectrum of the probe on the cytoplasmic side of the platelet membrane was also measured. Samples of 0.5 ml of platelet suspension were collected and the probe remaining in the medium and bound to the outside layer of the plasma membrane was washed out with albumin as described above, and resuspended in 0.5 ml Na,K-Tris for measurement of its fluorescence spectrum.

Results

Binding of pyrene derivatives to platelets

We first examined the binding to whole platelets of the butyltrimethylammonium derivative of pyrene and the other pyrene derivatives shown in Fig. 1. Pyrene derivatives have been used for measuring the fluidity of the lipid bilayer in biological membranes and model lipid membranes [9–13,15] and as markers of the effect of lipid peroxidation on biological bilayer membranes [16]. However, the relationships of the structures of pyrene derivatives and their binding affinities to membranes are still unknown. As shown in Fig. 2, the cationic butyltrimethylammonium derivative bound to the platelets immediately after its addition to a platelet suspension, and then its binding increased gradually.

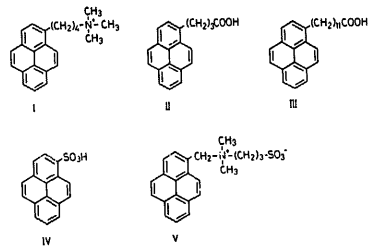

Fig. 1. Chemical structures of the butyltrimethylammonium derivative of pyrene and other pyrene derivatives tested in this study. I, 1-pyrenebutyltrimethylammonium; II, 1-pyrenebutanoic acid; III, 1-pyrenedodecanoic acid; IV, 1-pyrenesulfonic acid; V, 3-(*N,N*-dimethyl-*N*-(1-pyrenemethyl)ammonium)propanesulfonate.

After 4 h at 37°C the fraction bound to platelets was 66% of the total probe added and after 8 h it became 93% (data after 8 h not shown in Fig. 2). The rate of increase depended on the temperature, being much greater at 37°C than at 25°C. These characteristics of binding of the quaternary ammonium probe are consistent with those of TMA-DPH [7].

We also examined the bindings of other probes to whole platelets. Unlike the binding of the cationic probe, those of other derivatives did not change during incubation. As shown in Table I, of the derivatives tested, fatty acid derivatives, especially dodecanoic derivatives, bound to platelets markedly. The sulfonic acid derivative and zwitterionic derivatives bound much less, indicating that they are not good probes because for use as probes, pyrene derivatives must have high affinity to membranes as they are fluorescent in aqueous medium.

TABLE I

Binding of pyrene derivatives to platelets

Samples of platelet suspension were incubated with 1.5 μM concentrations of pyrene derivatives for 2 min at 37°C. Experimental procedures were as for Fig. 2. Data are means ± S.D. for four experiments. The roman numbers correspond to those of the pyrene derivatives shown in Fig. 1.

Probe	Bound probe (10^5 molecules/platelet)
II	1.82 ± 0.19
III	5.48 ± 0.18
IV	0.09 ± 0.02
V	0.35 ± 0.06

Transbilayer incorporation of the butyltrimethylammonium derivative

In previous studies on TMA-DPH, it was found that time-dependent increase in binding of the quaternary ammonium probe to platelets is due to its transbilayer incorporation [7,8]. Therefore, we next examined the incorporation of the butyltrimethylammonium derivative of pyrene into the inner membrane compartment. As shown in Fig. 3, incorporation of the cationic probe into the inner membrane compartment was gradual

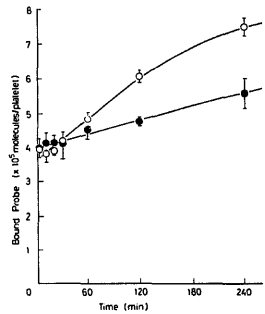

Fig. 2. Time courses of change in binding of the butyltrimethylammonium derivative to platelets during incubation at 37°C (○) and 25°C (●). A final concentration of $8 \cdot 10^5$ platelets/μl was incubated with 1.5 μM derivatives at each temperature. Then 0.5 ml of the suspension was collected and centrifuged. The fluorescence intensities of the probe in the supernatant and pellet were measured and bindings were calculated as described in Materials and Methods. Data are means ± S.D. for four experiments.

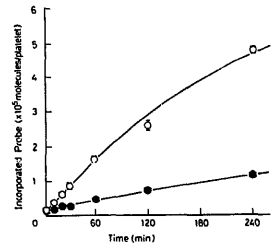

Fig. 3. Incorporation of the butyltrimethylammonium derivative into the inner membrane compartment at 37°C (○) and 25°C (●). Incorporation of the derivative into the inner membrane compartment was assayed by measuring increase of the fraction not extractable with albumin. After incubation with 1.5 μM derivative, platelets were washed twice with a solution of albumin and solubilized with SDS. The amount of incorporated probe was determined by measuring the fluorescence intensity. Data are means ± S.D. for four experiments.

and did not reach equilibrium even after 4 h. After 4 h at 37°C the fraction incorporated into the cytoplasmic side was 42% of total probe added and after 8 h it became 69% (data after 8 h not shown in Fig. 3). At 25°C the rate of incorporation was much less. These results are consistent with those on increases in the total binding shown in Fig. 2 and prove that the increase in total binding is due to the incorporation of the butyltrimethylammonium derivative into the inner membrane compartment. The data shown in Fig. 2 minus those in Fig. 3 indicate the binding of the probe to the outer leaflet of the plasma membrane. The data obtained by this calculation revealed that the ratio of the amount of the probe in the outside layer of the plasma membrane to that on the inner membrane compartment was 1.0:1.8 after 4 h and it became 1.0:2.9 after 8 h when most of the probe added had already bound to the platelets. Preferential binding of the probe to the cytoplasmic side is consistent with that for TMA-DPH reported previously [7,8]. However, the rate of transbilayer incorporation of the butyltrimethylammonium derivative of pyrene was much slower than that of TMA-DPH, although its affinity to the platelet membrane was rather more than that of TMA-DPH, possibly due to a difference in its molecular structure from that of TMA-DPH.

Change in binding of the butyltrimethylammonium derivative to the outer leaflet of the plasma membrane after platelet activation

The slow penetration of the quaternary ammonium probe described above suggests that this probe should be useful in studies on the physico-chemical properties of both the outer layer and the cytoplasmic side of the platelet membrane. In the initial stage, this probe was scarcely incorporated in the inner leaflet of the plasma membrane, allowing study of its binding to the outside of the plasma membrane without consideration of its incorporation into the membrane. Binding of this cationic probe to the outer leaflet of the plasma membrane is thought to be affected by change of the membrane surface potential [17]. Therefore, change in the surface potential of the platelet plasma membrane on activation of platelets could be examined by measuring the binding of the probe to the outer leaflet of the platelet plasma membrane. Platelet activation is reported to be associated with the appearance of the acidic phospholipid phosphatidylserine in the outer leaflet of the plasma membrane [18,19]. We investigated the effect of the Ca^{2+} ionophore ionomycin on the binding of the butyltrimethylammonium derivative to the outer leaflet of the platelet plasma membrane.

As shown in Table II, binding of the cationic probe to platelets (binding to the outer leaflet of the plasma membrane) increased on activation of the platelets with 5 μM ionomycin. About 30% increase of binding was observed in the presence of 10 μM ionomycin (data not shown in Table II). Although there is a possibility that the increase in membrane binding of the probe stemmed from the increase in its transbilayer movement as suggested by Bevers et al. [8], we confirmed that the increase was not due to the incorporation of the probe into the inner membrane compartment (data not shown). On the other hand, the bindings of membrane permeable fatty acid derivatives to platelets were not changed by activation of the platelets. The increase of the binding of the cationic probe to the outer membrane layer corresponded with decrease in the fluorescence intensity of the anionic, membrane impermeable probe 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS), which represents decrease of its binding to the outside layer of the plasma membrane (about 10% decrease in fluorescence intensity was observed in these conditions). These changes may correspond with the appearance of phosphatidylserine on the platelet surface mentioned above and associated change of membrane surface potential.

TABLE II

Effects of 5 μM ionomycin on binding of pyrene derivatives to platelets

Samples of platelet suspension as for Fig. 2 were incubated with 1.5 μM concentration of pyrene derivatives in the presence or absence of 5 μM ionomycin for 2 min at 37°C. Binding was assayed as described for Fig. 2. Binding of each derivative in the absence of ionomycin was defined as 1.00 and mean relative values ± S.D. in four experiments in the presence of ionomycin are listed. The roman numerals for pyrene derivatives correspond to those in Fig. 1. The statistical significance was determined by Student's *t*-test: *** $P < 0.001$.

Probe	Relative binding amount
I	1.18 ± 0.04 ***
II	0.98 ± 0.02
III	0.98 ± 0.04

Excimer formation of the incorporated butyltrimethylammonium derivative and its change on platelet activation

Pyrene derivatives are known to form excimers in membrane lipid bilayers and their formations have been measured in studies on membrane fluidity [9–13]. The rate of pyrene excimer formation in a phospholipid bilayer is reported to be related with the lateral diffusion coefficient of the probe [9,10]. Therefore, using a higher concentration of the cationic derivative, we next examined its excimer formation during its incorporation.

As shown in Fig. 4, just after the addition of 80 μM probe to the platelet suspension, a highly structured band (peak at 377 nm), which corresponds to the emission of the excited monomer [10], was observed but scarcely any excimer formation at longer wavelength was detected. At this stage, the fluorescence was that of the probe in the medium and that present in

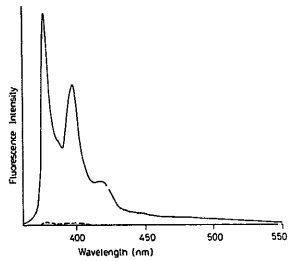

Fig. 4. Fluorescence spectra of 80 μM butyltrimethylammonium derivative after incubation with platelet suspension for 2 min at 37°C. The solid line shows the spectrum of whole probes in platelet suspension and the broken line shows the spectrum of probes on the cytoplasmic side. Platelets were incubated with 80 μM derivative for 2 min at 37°C before measurement of the fluorescence spectrum. The fluorescence spectrum of the probe on the cytoplasmic side was measured after washing the platelets with a solution of albumin and resuspending them in Na,K-Tris. The excitation wavelength was 342 nm.

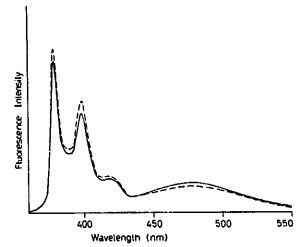

Fig. 6. Change of the fluorescence spectrum of the butyltrimethylammonium derivative incorporated into the cytoplasmic side by activation of platelets with 5 μM ionomycin. The solid line shows the control spectrum and the broken line shows the spectrum of the probe in the presence of ionomycin. After incubation with 80 μM derivative for 120 min at 37°C, the platelets were washed with albumin and resuspended in Na,K-Tris as described for Fig. 2. Then the suspension was incubated for 2 min at 37°C in the presence or absence of 5 μM ionomycin and fluorescence spectra were measured.

the outer leaflet of the plasma membrane. However, as shown in Fig. 5, after 120 min incubation of the probe with the platelets, a broad structureless band (peak at 479 nm) was seen, which represented emission of the excimer [10], and the intensity of emission of the monomer had decreased. The spectrum of the incorporated probe present in the inner membrane compartment, which is shown in Fig. 5, indicates that the excimer was formed by the probe incorporated into the cytoplasmic side. At this stage, the concentration of the probe on the cytoplasmic side was similar to that in the outer layer of the plasma membrane. Moreover, the spectrum due to excimer formation was observed earlier (even after 60 min; data not shown). These results suggest that on the cytoplasmic side the probe is in a condition in which it readily forms excimer, although it is difficult to conclude the lack of excimer formation in the outer layer only from these experimental results.

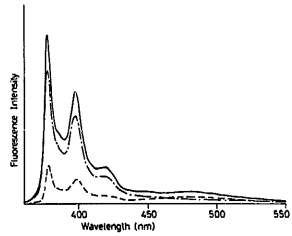

Fig. 5. Fluorescence spectra of 80 μM butyltrimethylammonium derivative after incubation with platelet suspension for 120 min at 37°C. The solid line shows the spectrum of probe in a whole platelet suspension, the broken line shows the spectrum of probes on the cytoplasmic side and the dot-dash line shows the difference spectrum. Experimental procedures were as for Fig. 4 except for the incubation time.

We next examined the effect of ionomycin on the fluorescent spectrum of the incorporated probe. The results in Fig. 6 show that after platelet activation, the ratio of excimer decreased. The ratio of the fluorescence intensity of excimer at 479 nm to that of monomer at 377 nm was 0.192 ± 0.011 for control platelets and 0.168 ± 0.005 for ionomycin-activated platelets (means $\pm$ S.D. for four experiments). This decrease could be due to decrease in fluidity near the probe. Another possibility is change in distribution of the cationic probe because its distribution may be heterogeneous due to possible heterogeneity in the distribution of phospholipids in the leaflets.

Discussion

Quaternary ammonium ions such as 1-pyrenebutyltrimethylammonium tested here seem to bind first to the outer layer of the plasma membrane and then gradually penetrate into the cytoplasmic side by a flip process. Theoretically, in equilibrium conditions, they should become bound preferentially to the cytoplasmic surface of the membranes which has a negative surface potential due to the presence of acidic phospholipids

[20]. For example, in erythrocytes, quaternary alkyl ammonium ions have been shown to flip slowly to the inner leaflet and change the initial echinocytogenic shape to a stomatocytogenic shape by preferentially binding to the inner layer [21]. Likewise, the results shown in Figs. 2 and 3 show that the butyltrimethylammonium derivative of pyrene bound preferentially to the cytoplasmic surface of the membranes in equilibrium conditions.

The characteristic of the butyltrimethylammonium derivative of pyrene of slow penetration through the plasma membrane is favorable for use of this derivative to study the physico-chemical properties of both the outer layer of the plasma membrane and cytoplasmic side of platelet membranes and their changes. The observation that formation of excimer of the probe was associated with its incorporation into the inner membrane compartment suggests that the lipid environment near the cationic probe on the cytoplasmic side of platelet membranes is more fluid than that in the outside layer. This possibility is supported by previous results on the fluorescence anisotropy of TMA-DPH [7]. However, our findings do not exclude the possibility that binding sites of the probe on the cytoplasmic side are more condensed than those on the outside layer and that excimer formation is facilitated by a possible heterogeneous distribution of lipids in the leaflets. Moreover, the binding sites of the probe on the cytoplasmic side may be complex. As shown in fibroblasts [13], some proportions of cationic probes penetrating to the cytoplasmic side seem to bind to organella membranes. Excimer formation may also stem from binding of the probes to intrinsic proteins which would restrict their lateral mobility.

Significant loss of membrane phospholipid asymmetry occurs when platelets are stimulated with a Ca^{2+} ionophore [19]. This loss of asymmetry is probably related to the observed changes in fluorescence properties, namely, the increase in the binding of the probe to the outside layer of the plasma membrane and change in the fluorescence spectrum of the probe on the cytoplasmic side of the platelet membranes, because phosphatidylserine appears in the outer layer possibly resulting in loss of asymmetry of membrane fluidity.

Bevers et al. revealed that ionomycin stimulated rapid flip-flop of TMA-DPH in human platelets which induced more than three times increase in total amount of its membrane binding [8]. We also observed that ionomycin induced an about 2-fold increase in fluorescence intensity of the same probe in bovine platelets [7]. Since the probe incorporated into the inner membrane compartment seems to be easily extracted with albumin when rapid flip-flop occurs. Therefore, it seems to be difficult for TMA-DPH to clearly distinguish the increase in binding to the outer membrane layer from the increase in transbilayer incorporation, which may have caused discrepancies in the results of the previous papers [7,8]. The transbilayer incorporation rate of 1-pyrenebutyltrimethylammonium is much lower than that of TMA-DPH. Therefore, for the cationic pyrene derivative, ionomycin-induced increase in its binding to platelets probably reflects only the increase in its binding to the outer leaflet of the plasma membrane. These things may be the reason why ionomycin-induced increase in binding of the pyrene derivative to platelets was much less than that of TMA-DPH [7,8].

The transport mechanism of quaternary ammonium ions through biological membranes is still unknown. Transport is significantly temperature-dependent, suggesting the presence of a large energy barrier. Some investigators have suggested that various quaternary ammonium ions permeate through the lipid bilayer as lipophilic ion-pairs with endogenous anions [22]. Recently TMA-DPH has been suggested to be internalized by L929 cells and macrophages by endocytosis [23]. Because of the slow penetration of quaternary ammonium probes, two conditions can be distinguished: that in which the probes are localized in the outer leaflet of the plasma membrane and that in which they become localized in the cytoplasmic side of the platelet membranc after albumin washing. Therefore, these quaternary ammonium probes seem to be useful for studies on the physico-chemical properties of both sides of biological membranes and their changes.

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