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Permalink

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Journal

Platelets, 5(3)

ISSN

0953-7104

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Publication Date

1994

DOI

10.3109/09537109409005527

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Peer reviewed

Probing the Interaction of PAF with Human Platelet Membrane Using the Fluorescent Probe Laurdan

A. Kantar, P. L. Giorgi, E. Gratton, R. Fiorini

SUMMARY. Changes in membrane polarity of human platelets during the interaction with PAF were investigated by measuring the steady-state fluorescence emission spectra of 2-dimethylamino(6-lauroyl)naphthalene (Laurdan), which is known to be incorporated at the hydrophobic-hydrophilic interface of the bilayer, displaying spectral sensitivity to the polarity of its surroundings. Laurdan shows a marked steady-state emission red-shift in polar solvents, with respect to non-polar solvents. Our results demonstrate that platelet activation factor (PAF) (10^{-7} M) induces a red-shift of the fluorescence emission spectra of Laurdan. These changes were not observed in the presence of the PAF antagonist, L-659,989. These data suggest that the interaction of PAF with its specific receptor and the signalling pathways involved in platelet activation are accompanied by an increase in polarity at the hydrophobic-hydrophilic interface of human platelet membranes.

Platelet activating factor (PAF, 1-O-alkyl-2-O-acetyl-sn-glycerol-3-phosphocholine) is a potent lipid mediator with a wide spectrum of biological activities in a variety of cells.¹⁻³ PAF exerts its actions through the activation of specific membrane receptors.⁴ Human platelets possess a specific extracellular PAF-receptor.^{5,6} The stimulation of human platelets by PAF is accompanied by a complex cascade of biochemical events that influence the plasma membrane.⁷ These events, initiated by the binding of PAF to its specific receptor, include the stimulation of GTPase,⁸ transient elevation of intracellular Ca^{2+} ,⁹ coupling to both adenylate cyclase and phospholipase C,¹⁰ and protein phosphorylation.¹¹ The response of platelets to PAF is transient and PAF desensitizes its receptor.¹² Fluorescence spectroscopy techniques have been widely employed to study the physico-chemical changes in membrane organization. The

fluorescent membrane probe 2-dimethylamino (6-lauroyl)naphthalene (Laurdan) displays spectral sensitivity to the polarity of its surrounding, showing a red-shift of the emission in polar solvents, with respect to non-polar solvents and a blue-shift in non-polar with respect to polar solvents.¹³

In this study we investigated the effect of PAF on membrane polarity of platelets, by measuring steady-state emission spectra of Laurdan incorporated into the platelets plasma membrane before and after the addition of PAF in the absence or presence of the PAF antagonist L-659,989.¹⁴

Materials and Methods

Preparation of Platelets

10 ml of freshly drawn acid-citrate-dextrose anticoagulated blood was centrifuged at 120 g for 15 min at 37°C to prepare the platelets rich plasma. The platelets were isolated and washed according to Kubina et al.¹⁵ The platelets were finally resuspended in Tyrode's buffer (137 mM NaCl, 2 mM KCL, 12 mM NaHCO₃, 0.3 mM NaH₂PO₄, 1 mM MgCl₂,

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2 mM CaCl₂, 5 mM glucose) with 0.1% bovine serum albumin and apyrase (Sigma Chemical Co., St. Louis, MO, USA). The total platelet count was obtained using a Sysmex E-2500 blood analyser. The platelet suspension was adjusted to a concentration of 4×10^8 /ml. Phase contrast microscopy (Diaplan, Leica, Milan, Italy) showed that, under the conditions used, control and Laurdan labelled platelets remained discoid and functional, being sensitive to thrombin (1 U/ml).⁷ The platelets were immediately used for fluorescence measurements.

Chemical Agents

6-dodecanoyl-2-dimethylamino-naphthalene, Laurdan was purchased from Molecular Probes Inc. (Eugene, OR, USA) and PAF from Sigma Chemical Co. (St. Louis, MO, USA). L-659,989 was kindly donated by Dr W. H. Parsons, Merck Sharp & Dohme Research Laboratories, NJ, USA.

Fluorescence Measurements

The platelets were labelled with an ethanol solution of Laurdan under N₂ at a final probe concentration of 0.1 μM. All samples were prepared at room temperature and used immediately for fluorescence measurements. Steady-state excitation and emission spectra were measured at 37°C before and during activation of the platelets, with a Perkin Elmer Spectrofluorimeter MPF66, equipped with a Perkin Elmer 7300 Personal Computer for data acquisition and elaboration as previously described.¹⁶ The platelets were activated by injecting PAF (10^{-7} M) into the sample and fluorescence spectra were immediately measured. PAF antagonist, L-659,989 was added at a final concentration of 10^{-6} M before activation with PAF. Fluorescence measurements were performed in ten samples.

Results

The background phospholipid fluorescence of the platelets was checked prior to each measurement and was less than 0.1% of the fluorescence when Laurdan was added. The contribution of the light scattering was negligible in our samples because of the low cell concentration used in this study. The fluorescence intensity of the free probe in buffer solution, in the absence of platelets, was negligible and did not increase upon the addition of PAF at the concentration used in the study; moreover, this concentration (10^{-7} M) is lower than the critical micelle concentration of PAF (2×10^{-5} M) as reported in the literature.¹⁷ After the addition of 0.1 μM Laurdan to the platelets a constant increase in fluorescence was observed, plateauing after 10 min (data not shown). Dilution experiments, which are based on the rapid equilibrium of Laurdan between plasma membrane and buffer solution, have demonstrated

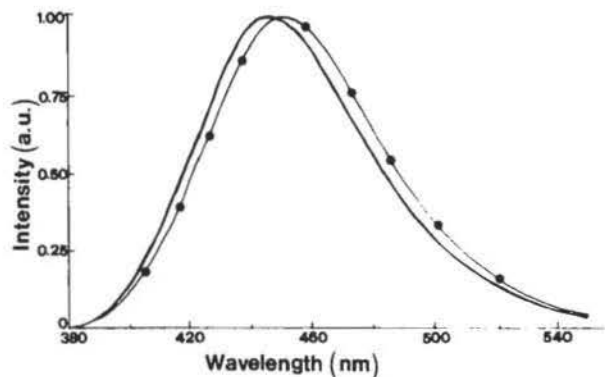


Fig. 1 Normalized emission spectra of Laurdan incorporated in the platelet plasma membrane at 37°C before (—) and after (---●) addition of PAF 10^{-7} M. The spectra were measured at an excitation wavelength of 350 nm.

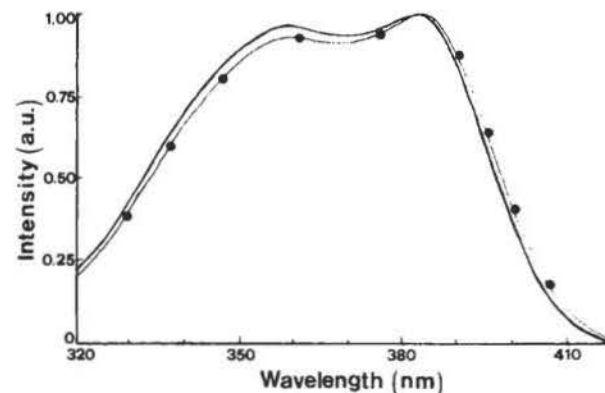


Fig. 2 Normalized excitation spectra of Laurdan incorporated in the platelet plasma membrane at 37°C before (—) and after (---●) addition of PAF 10^{-7} M. The spectra were measured at an emission wavelength of 450 nm.

that the probe in platelets is located in the plasma membrane during the acquisition of the measurements.

Fluorescence emission and excitation spectra of Laurdan in platelets before and during activation with PAF are reported in Figures 1 and 2, respectively. During activation with PAF (Fig. 1) a 5 nm red-shift of the Laurdan emission spectrum was observed. During basal conditions the maximum of Laurdan emission was at 446 nm, while after activation with PAF it was at 451 nm.

The Laurdan excitation spectra during activation of the platelets with PAF (Fig. 2) showed a decrease in intensity at shorter wavelengths with respect to the excitation spectrum in basal conditions. The spectral changes induced by PAF were observed in all samples. The PAF antagonist L-659,989 had no effect on emission or excitation spectra of Laurdan in unactivated platelets.

In the presence of L-659,989, the addition of PAF did not change fluorescence spectra significantly.

Discussion

The organization of cell membrane components depends on a number of factors, including the

protein-lipid composition of the membrane, receptor occupancies, and activities of the membrane proteins.¹⁸ Cell membrane respond to stimuli by activation of membrane components that lead to signal transduction. These events lead to changes in membrane composition and configuration. Depending on the nature of the stimulus and the state of the membrane, different biochemical events take place at the membrane level during activation of the cell.^{19,20}

The fluorescent probe Laurdan has been recently employed to detect modifications in the composition and physical state of living cells.^{17,21-23} Laurdan has been reported to be incorporated at the hydrophilic-hydrophobic interface of the membrane, with the lauric acid tail anchored in the hydrophobic region of the bilayer.²⁴ It has been demonstrated that Laurdan displays spectral sensitivity to the polarity of its surroundings, showing a red-shift of the emission in polar solvents, with respect to nonpolar solvents.¹³ This behavior is referred to dipolar relaxation phenomena that are related to the physical state and the dynamics of the surrounding phospholipid polar head group.²⁵ In single-phase phospholipid vesicles the dynamics of the surroundings detected by Laurdan is very different in the case of the gel or of the liquid-crystalline phase; the probe shows a marked steady-state emission red-shift in the phospholipid liquid-crystalline phase, with respect to the gel phase. If solvent molecules can move during the fluorescence lifetime, the Laurdan excited-state molecular dipole will orient the neighbouring solvent dipoles, and the steady-state Laurdan emission spectra will be red-shifted.²⁶ On the other hand, Laurdan undergoes a marked blue-shift when incorporated in the gel-crystalline phase, with respect to the liquid phase.²⁶

Our results demonstrate that PAF induced a red-shift of the emission spectra of Laurdan incorporated in platelet membranes, indicating an increase in polarity of the environment surrounding the probe. In basal condition, Laurdan reflects the interactions between the probe and the phospholipid polar head groups. The addition of PAF to platelets increases the motional freedom of the bilayer polar residues, as demonstrated by the presence of an unrelaxed red-shift of the spectrum. In a previous study, using the fluorescent probe 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), we have shown that PAF induces a time limited increase in platelet membranes phospholipid packing and a decrease in membrane heterogeneity.⁷ Thus, it is therefore possible that such changes may alter the hydrophobic environment surrounding the Laurdan molecules reflecting an increase in membrane polarity. It is interesting that our previous studies demonstrated that PAF induces a decrease in plasma membrane polarity of human neutrophils.²¹ The differences in the PAF receptor, G proteins and effectors between human platelets and human neutrophils have been reviewed by Hwang.⁵ It is plausible

that the distinct molecular events accompanying the activation of human platelets or neutrophils modulates membrane polarity differently. Our data demonstrated that the anti-PAF L-659,989 blocked the effect of PAF on membrane polarity suggesting that these changes may be attributed to the PAF-receptor interaction and the biochemical events associated with signal transduction.

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