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USE OF LAURDAN FLUORESCENCE IN STUDYING PLASMA MEMBRANE ORGANIZATION OF POLYMORPHONUCLEAR LEUKOCYTES DURING THE RESPIRATORY BURST*

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Abstract—The changes in plasma membrane polarity of polymorphonuclear leukocytes (PMN) during the activation of the respiratory burst 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 blue shift in nonpolar solvents, with respect to polar solvents. Our results show a blue shift of the fluorescence emission spectra of Laurdan during activation of PMN with phorbol myristate acetate or *N*-formyl-methionyl-leucyl-phenylalanine. These results suggest that the activation of the respiratory burst of PMN is accompanied by a decrease in polarity in the hydrophobic–hydrophilic interface of the plasma membrane.

INTRODUCTION

The activation of the respiratory burst of polymorphonuclear leukocytes (PMN)‡ is accompanied by a complex cascade of biochemical events that influence the plasma membrane.[1–3] These events include ligand–receptor interaction, activation of membrane G-protein, membrane cytoskeleton interactions, activation of membrane phospholipase, protein phosphorylation and translocation of cytosol proteins to the membrane level.[4–6] Although the stimulation pathways employed by various agents are coupled to the activation of the respiratory burst oxidase, the details and mechanisms involved are still unknown. The respiratory burst oxidase is an electron transport system that catalyzes the one electron reduction of oxygen to produce superoxide anion at the expense of reduced nicotinamide adenine dinucleotide phosphate (NADPH).[2,7]

Fluorescence spectroscopy techniques have been widely used to study the physico-chemical changes of membrane organization.[8–10] The fluorescent membrane probe Laurdan (2-dimethylamino[6-lauroyl] naphthalene) is known to be sensitive to the polarity of the environment, displaying spectral sensitivity to the phospholipid phase state.[11]

In this study we have investigated changes in plasma membrane polarity of PMN during the activation of the respiratory burst by measuring steady-state emission spectra of Laurdan incorporated into the PMN plasma membrane during stimulation with phorbol myristate acetate (PMA) or *N*-formyl-methionyl-leucyl-phenylalanine (fMLP).

MATERIALS AND METHODS

Preparation of PMN. Human PMN were isolated from freshly drawn blood of 10 healthy volunteers using a Mono-Poly Resolving Medium (Flow Laboratories) as previously described.[12] Cells were suspended in Krebs'–Ringer phosphate (KRP) solution supplemented with 1 mg/mL glucose at a final concentration of 10^4 PMN/mL and 10^6 PMN/mL for chemiluminescence and fluorescence studies, respectively.

Chemical agents. Laurdan, 6-dodecanoyl-2-dimethylamino naphthalene was purchased from Molecular Probes Inc. (Eugene, OR). Luminol, PMA and fMLP were obtained from Sigma Chemical Co. (St. Louis, MO).

Chemiluminescence measurements. Luminol amplified chemiluminescence was measured in an LKB RackBeta 1211 beta-counter and PMN (in the absence or presence of Laurdan) were activated by addition of PMA (1.5×10^{-6} *M*) and fMLP (10^{-5} *M*) as previously described.[3,12]

Fluorescence measurements. Polymorphonuclear leukocytes were labeled with an ethanol solution of Laurdan under N_2 at a final probe concentration of 0.05 μM. All samples were prepared at room temperature and in red light and used immediately for fluorescence measurements. Steady-state excitation and emission spectra were measured at 37°C before and during activation of PMN on a photon counting spectrofluorometer (model GREG PC, ISS Inc., Urbana, IL) and using the ISS Inc. software. The actual temperature was measured in the sample cuvette using a digital thermometer. Polymorphonuclear leukocytes were activated by addition of stimulating agents PMA (1.5×10^{-6} *M*) or fMLP (10^{-7} *M*) as described elsewhere.[3]

RESULTS

Chemiluminescence studies

Luminol-amplified chemiluminescence has been employed, as previously described[3] to verify that in isolated PMN the superoxide-generating oxidase system is dormant under resting conditions and can be activated by PMA and

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‡*Abbreviations:* fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; KRP, Krebs'–Ringer phosphate solution; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocytes; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene.

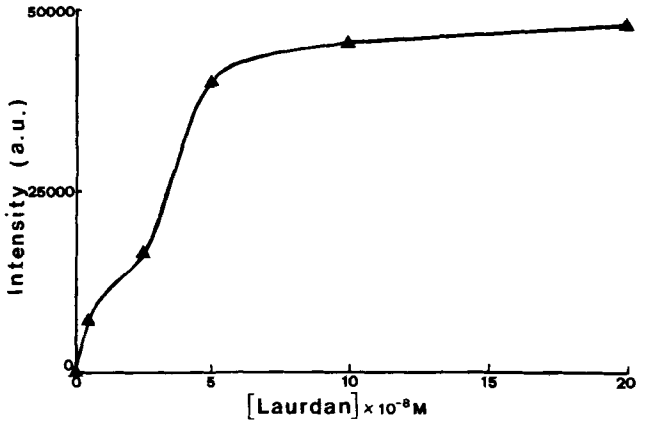

Figure 1. Fluorescence intensity of Laurdan incorporated in polymorphonuclear leukocyte (PMN) plasma membranes as a function of Laurdan concentration at 37°C.

fMLP. All samples used in this study demonstrated an activatable NADPH-oxidase system (data not shown). Polymorphonuclear leukocytes labeled with Laurdan 0.05 μM still showed a normal burst.

Fluorescence studies

The background phospholipid fluorescence of PMN 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 KRP, in the absence of PMN, was negligible and did not increase upon the addition of PMA or fMLP at the concentrations used in the study. Moreover, the excitation and emission spectra of Laurdan in KRP did not change after the addition of PMA or fMLP.

The fluorescence intensity of Laurdan incorporated in PMN (10^6 cells/mL) as a function of probe concentration was measured to establish the optimal concentration of Laurdan for the experiments performed. As shown in Fig. 1, an increase in probe concentration was accompanied by a proportional increase in fluorescence intensity up to saturation at 0.05 μM. After the addition of 0.05 μM Laurdan to PMN a constant increase in fluorescence was observed, plateauing after 10 min (Fig. 2). Dilution experiments have demonstrated that Laurdan in unstimulated and stimulated PMN is located in the plasma membrane. These experiments are based on the rapid equilibrium of Laurdan between plasma membranes and KRP solution.

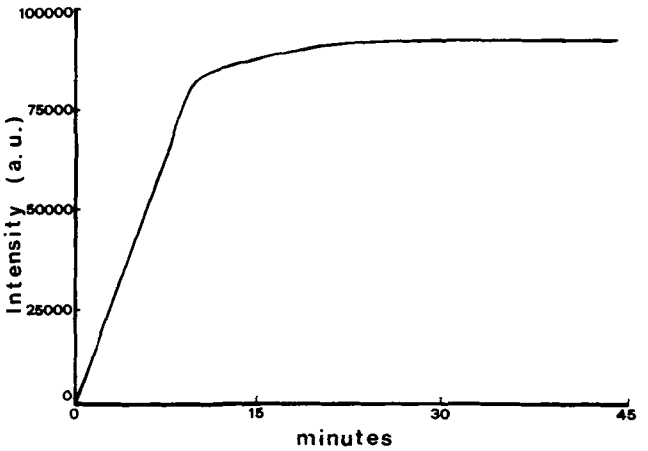

Figure 2. Kinetic of incorporation of Laurdan (0.05 μM) in PMN plasma membranes monitored by the increase in total fluorescence as a function of time at 37°C.

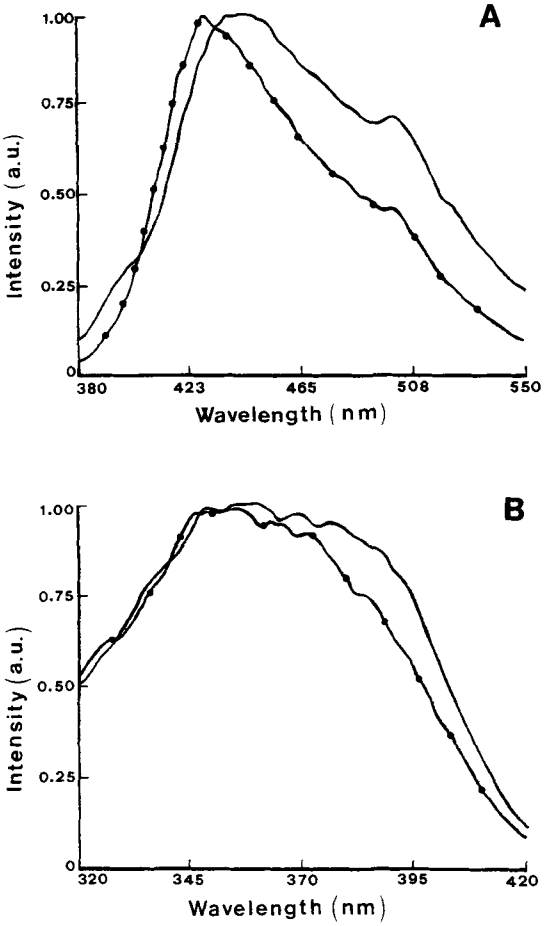

Figure 3. (A) Normalized emission spectra of Laurdan incorporated in PMN plasma membranes at 37°C before (——) and during activation with 1.5×10^{-6} M PMA (-●-●-). The spectra were measured at an excitation wavelength of 350 nm. (B) Normalized excitation spectra of Laurdan incorporated in PMN plasma membranes at 37°C before (——) and during activation with 1.5×10^{-6} M PMA (-●-●-). The spectra were measured at an emission wavelength of 450 nm.

Fluorescence emission and excitation spectra of Laurdan in PMN before and during activation with PMA and fMLP are reported in Figs. 3 and 4, respectively. During activation with PMA (Fig. 3A) a blue shift of the Laurdan emission spectrum (maximum at 428 nm) with respect to emission in basal conditions (maximum at 443 nm) was observed. Also fMLP (Fig. 4A) induced a blue shift (maximum at 435 nm) with respect to basal conditions (maximum at 443 nm).

The Laurdan excitation spectra during activation of PMN with PMA (Fig. 3B) or fMLP (Fig. 4B) showed a decrease in intensity at longer wavelengths with respect to the excitation spectrum observed in basal conditions.

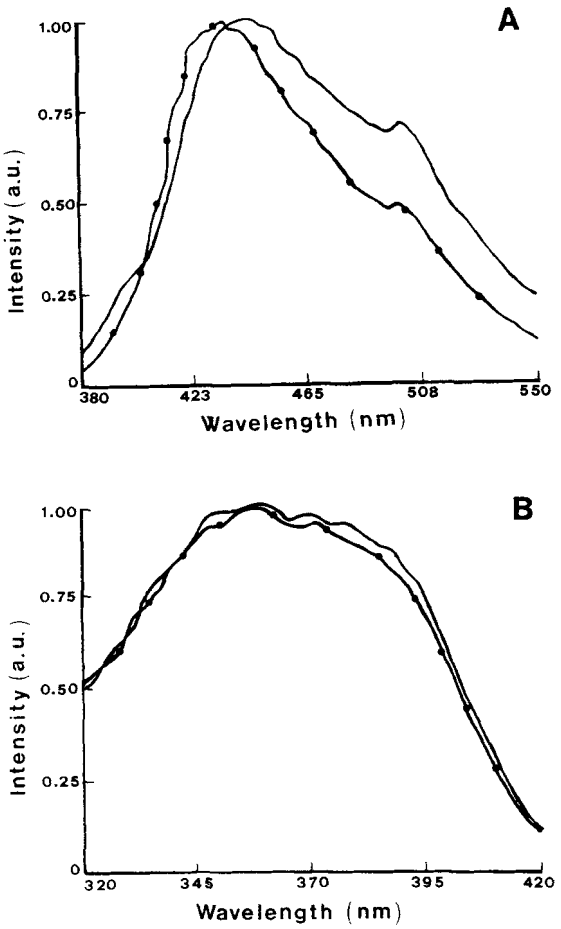

Figure 4. (A) Normalized emission spectra of Laurdan incorporated in PMN plasma membranes at 37°C before (——) and during activation with 10^{-7} *M* fMLP (-●-●-). The spectra were measured at an excitation wavelength of 350 nm. (B) Normalized excitation spectra of Laurdan incorporated in PMN plasma membranes at 37°C before (——) and during activation with 10^{-7} *M* fMLP (-●-●-). The spectra were measured at an emission wavelength of 450 nm.

DISCUSSION

The fluorescent probe Laurdan has been reported to be incorporated at the hydrophilic-hydrophobic interface of the membrane[13] with the lauric acid tail anchored in the hydrophobic region of the bilayer. It has been demonstrated that Laurdan displays spectral sensitivity to the polarity of its surrounding, showing a red shift of the emission in polar solvents, with respect to nonpolar solvents.[11] 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.[14] 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.[11] If solvent molecules can move during the fluorescence lifetime, the Laurdan excited-state molecular dipole will orient the neighboring solvent dipoles, and the steady-state Laurdan emission spectrum will be red shifted.[11,15]

Our results demonstrate that the activation of the respiratory burst of PMN by PMA or fMLP is accompanied by a blue shift of the emission spectra of Laurdan, indicating a decrease in polarity of the environment surrounding the probe. The observed changes in polarity occur regardless of the stimulus used.

In basal conditions, Laurdan emission reflects the interactions between the probe and the phospholipid polar head groups. The activation of PMN dramatically decreases the motional freedom of the bilayer polar residues, as demonstrated by the presence of an unrelaxed blue shift of the spectrum. In a previous study, using 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) steady-state fluorescence anisotropy, we have shown an increase in PMN plasma membrane phospholipid packing during the activation of the respiratory burst.[3] This increase may cause a reduction in water penetration in the hydrophobic environment surrounding the Laurdan molecules, thus inducing a decrease in polarity. Phorbol myristate acetate and fMLP have different biochemical pathways of activation and they initiate a series of biochemical events at the PMN plasma membrane level. Therefore, it seems likely that these biochemical events result in a decrease in membrane polarity.

This study demonstrates that the possible use of Laurdan is not only limited to model membranes but can be extended to living cells to follow functional responses.

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