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# Increased membrane heterogeneity in stimulated human granulocytes

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TMA-DPH fluorescence decay in human PMN before and after stimulation with FMLP was studied using frequency domain fluorometry. Membrane heterogeneity was assessed by the width of the continuous distributions of lifetime values of Lorentzian shape used to describe the fluorescence decay. In non-stimulated granulocytes TMA-DPH fluorescence decay is characterized by two distributions of lifetime values centered at 6.5 and 1.0 ns and full width at half maximum of 0.3 and 1.2 ns, respectively. Within 15 min after stimulation, the center values of the two distribution components were 5.1 and 0.8 ns and the distribution width was 0.8 and 0.6 ns, respectively. These results indicate changes of membrane domain organization which can be ascribed to compositional changes and redistribution of membrane components.

Membrane heterogeneity; Microdomain; Fluorescence lifetime; 1-(4-Trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene; (Granulocyte)

## 1. INTRODUCTION

Interaction of FMLP with specific membrane receptors induces activation of PMN functions occurring after rapid modifications of membrane lipid composition during the first 15 min after stimulation [1–3]. After these events the membrane recovers its initial lipid composition.

In a previous paper [4] using TMA-DPH fluorescence polarization, we have shown that a decrease of membrane fluidity in stimulated PMNs was detectable only in cells with disrupted microtubules [5], leading us to hypothesize that cytoskeleton affects the physico-chemical state of

the membrane, modulating the distribution of membrane components in the membrane plane.

To further analyse the involvement of membrane lateral organization in the stimulation process, we studied the changes of membrane heterogeneity using frequency domain fluorometry. We have shown that using an analysis method based on continuous distribution of lifetime values a description of membrane microheterogeneity can be obtained in model and natural membranes [6,7]. As fluorescent probe, we used TMA-DPH which is known to remain localized randomly in the plasma membrane for up to 25 min after its addition [8], enabling us to study membrane organization in intact cells.

## 2. MATERIALS AND METHODS

Human PMN cells were isolated from venous blood of healthy volunteers using Ficoll-Hypaque gradient centrifugation [9]. Cells were washed three times and suspended at a concentration of  $10^6$ /ml in HBSS supplemented with 5 mM glucose and were labeled with TMA-DPH (Molecular Probes Inc., Eugene, OR), final concentration  $10^{-6}$  M, as previously

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*Abbreviations:* PMN, polymorphonuclear leukocyte; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; HBSS, Hanks' balanced salt solution without red phenol

described [4]. After isolation, PMN cells were stimulated by addition of the chemotactic peptide FMLP (Sigma, St. Louis, MO, USA) at a concentration of  $10^{-7}$  M in HBSS. Fluorescence measurements were performed during the subsequent 10 min. To follow the effect of the stimulation as function of time we prestimulated PMNs ( $10^{-7}$  M FMLP in HBSS) and we added TMA-DPH after 15, 30, 45, 60 min at  $37^{\circ}\text{C}$ . No decrease in viability was observed using the Trypan blue exclusion test in all samples.

Lifetime measurements were performed at  $37^{\circ}\text{C}$  with a GREG 200 phase and modulation fluorometer (ISS Inc., Urbana, IL, USA), using a large set of modulation frequencies from 2 to 130 MHz. The wavelength of excitation was set at 325 nm (UV line of an HeCd laser, Liconix model 4240 NB). The experimental conditions of fluorescence measurements, the least-square routine for a multiexponential decay and the method for the derivation of a continuous distribution of lifetime values have been discussed elsewhere [6,10]. The distribution used in this work is characterized by a Lorentzian shape centered at a decay time  $C$  and having a full width at half maximum (FWHM). The values of the distribution widths obtained in this work are well within the resolvability limits established using simulated data for similar conditions [11,12].

### 3. RESULTS

Fig.1A and B shows the results of the lifetime distribution analysis of TMA-DPH decay in non-stimulated and stimulated PMN cells, respectively. Superimposed in fig.1 are the lifetime values obtained using a two-exponential analysis. In non-stimulated PMN, by using both analysis methods, two components were found (fig.1A): a long component with a lifetime (or the center) value of 6.5 ns and fractional intensity of around 0.8 and a short component with a lifetime (or the center) value of around 1 ns and with a fractional intensity of 0.2. The widths of the distribution were 0.3 ns for the long component and 1.2 ns for the short one. Within 15 min after the stimulation (fig.1B) the lifetime (or the centers) values of the long and short components were 5.1 and 0.8 ns and the distribution widths 0.8 and 0.6 ns, respectively. The relative fractional intensity of the two components did not change significantly upon stimulation. The distribution analysis with respect to the exponential one gives a further parameter, the width, which is remarkably modified by stimulation; moreover this analysis gives a better description of the TMA-DPH heterogeneous decay reaching a lower value of the reduced chi-squared (average chi-squared value reduction of about 45%; table 1). In fig.2A and B the changes of the FWHM value and of the center value of the

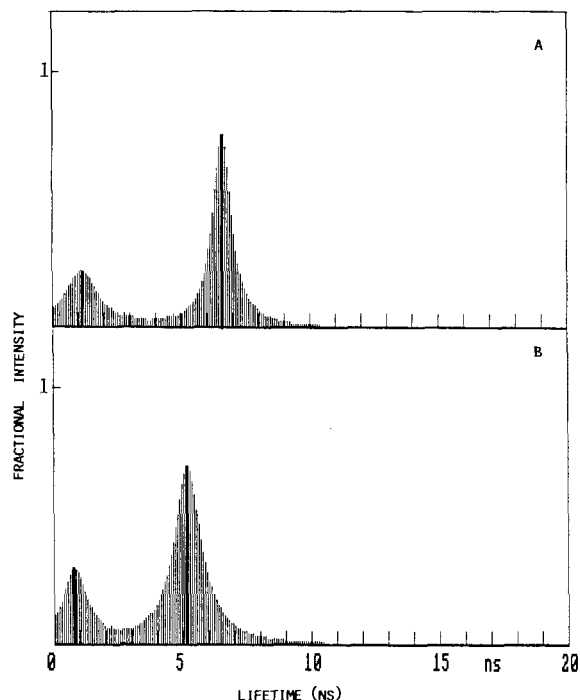


Fig.1. (A) DPH lifetime distribution in non-stimulated PMN cells. The distribution width of the long component has a value of 0.3 ns and is centered at 6.5 ns. (B) DPH lifetime distribution in stimulated PMN cells. The distribution width of the long component has a value of 0.8 ns and is centered at 5.1 ns.

distribution as a function of time after stimulation are reported. Error bars correspond to a change in the parameter which causes a 20% increase of the reduced chi-squared value. A decrease of the main component center value within 15 min is accompanied by a significant increase of FWHM. Successively the center value returned to the initial value while the FWHM value decreased slightly and did not reach the original value (fig.2A). The short component showed a slight change in the

Table 1

Reduced chi-squared values obtained with exponential and distribution analyses

	Exponential analysis	Distribution analysis
Non-stimulated PMN cells	3.3	1.5
Stimulated PMN cells	7.6	5.5

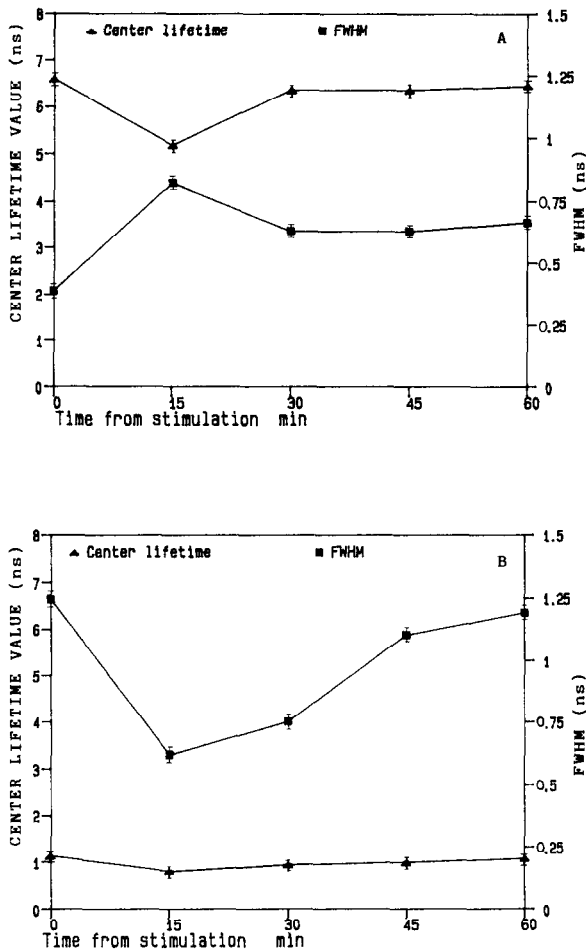


Fig.2. (A) FWHM (■) and center values (▲) for the long component of DPH lifetime distribution as a function of time after stimulation. (B) FWHM (■) and center values (▲) for the short component of DPH lifetime distribution as a function of time after stimulation. Error bars correspond to change of the parameter which causes a 20% increase of the reduced chi-squared.

center value and a significant decrease of FWHM 15 min after stimulation, recovering thereafter the initial values (fig.2B).

#### 4. DISCUSSION

The spectroscopic features of TMA-DPH can be referred to those of DPH [13] whose fluorescence lifetime value is dependent on the dielectric constant of the environment where the probe is located. Moreover we have shown that the lifetime

distribution width can be related to the degree of membrane heterogeneity [4,6]. Since TMA-DPH is anchored to the membrane surface with its cationic residues, the distribution of lifetime values can be related to the heterogeneity of membrane lateral organization. The distribution width is broader than that of DPH which can freely diffuse perpendicular to the membrane and average different environments during its excited state lifetimes [6]. The origin of the short lifetime component is still debated. In fact for DPH this component has been referred to photochemical derivatives of the probe [14] or alternatively it can represent a fraction of probe molecules localised at the surface in a very polar environment or in the boundary between environments of different physico-chemical features where water permeability is increased [15]. The change in the center of the lifetime distribution obtained with TMA-DPH in non-stimulated and stimulated PMNs 15 min after stimulation suggest a change in the average dielectric properties of the membrane. Following stimulation, an increase in the distribution width of the long component can be ascribed to an increase in membrane heterogeneity. During the first 5–10 min following stimulation the observed changes may concern compositional modifications [1,2]. However the changes of the heterogeneity pattern remain up to 60 min indicating that heterogeneity is dependent not only on lipid composition, but more in general, on the remodelling of membrane organization which follows the activation processes [16,17]. Our results indicate that TMA-DPH decay, when analysed using continuous lifetime distributions, is sensitive to modifications induced by activation.

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