Changes of fluorescence anisotropy in plasma membrane of human polymorphonuclear leukocytes during the respiratory burst phenomenon

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Received 18 August 1990; revised version received 4 September 1990

Steady state fluorescence anisotropy (r_s) of TMA-DPH was measured to study the effect of respiratory burst activation with PMA, FMLP, and PAF on the physico-chemical structure of PMNs plasma membrane. Our results show a significant increase in r_s during the respiratory burst activation. In the presence of NADPH-oxidase inhibitor DPI, only PAF induces changes in r_s values. This suggests a non-specific effect of PAF on plasma membrane. Azide, which induces a supranormal release of H_2O_2, fails to increase the basal r_s value after activation. Moreover, the catalase does not abolish the increase in r_s induced upon activation. This rules out the possibility that changes of r_s during the respiratory burst activation are attributed mainly to H_2O_2 release. We conclude that multiple processes accompanying the respiratory burst activation are responsible for the changes in the physico-chemical properties of PMNs plasma membrane.

Fluorescence anisotropy; TMA-DPH; Respiratory burst; PMN

1. INTRODUCTION

The activation of the respiratory burst (RB) is initiated by the stimulation of the membrane-bound NADPH oxidase system to generate superoxide anion (O_2^-) [1]. This system is activated by a wide variety of stimuli [2]. The biochemical events following the oxidase activation are multiple and complex, involving a number of processes which take place at the level of cell surface, plasma membrane and cytosol [2–6]. Several modifications of the plasma membrane fluidity of PMNs have been described in association with the RB [7–10]. Fluidity is a complex physico-chemical feature which depends upon the mobility and order of membrane components [11]. Changes in composition and molecular organization are the principal determinants inducing alterations in the physico-chemical structure of membrane.

In this study we have investigated the changes of steady-state fluorescence anisotropy (r_s) in PMNs during stimulation with various agents known to activate the RB. As fluorescent probe we have used 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), which goes to the lipid-water interface region of the plasma membrane [12], where it persists for up to 30 min [13], enabling us to detect physico-chemical changes of plasma membrane in living cells during the RB activation. Measurements have also been performed in the presence of RB inhibitors.

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Abbreviations: PMNs, polymorphonuclear leukocytes; TMA-DPH 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene; PMA, phorbol myristate acetate; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; PAF, 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; DPI, diphenyl iodonium

2. MATERIALS AND METHODS

2.1. *Preparation of PMNs*

Heparinised blood was obtained from 20 healthy non-smokers (18–30 years old) without any signs of acute infection or immunological disorders. PMNs were isolated using a Mono-Poly Resolving Medium (Flow Laboratories) as previously described [14]. Cells were resuspended in Krebs-Ringer phosphate solution plus 1 mg/ml glucose at a final concentration of 10^4 cells/ml and 10^6 cells/ml for chemiluminescence and fluorescence studies, respectively.

2.2. *Stimulating agents*

Phorbol myristate acetate (PMA), *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) and 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.3. *Respiratory burst inhibitors*

Diphenyl iodonium (DPI) (50 mM), sodium azide (1 mM) and catalase (2000 U/ml) were purchased from Sigma. The inhibitors were added to PMNs before activation.

2.4. *Chemiluminescence studies*

Luminol amplified chemiluminescence was measured in an LKB RackBeta 1211 beta-counter as previously described [14].

After measuring the basal chemiluminescence level for 15 min, PMNs were activated by addition of 1.5×10^{-6} M PMA, 10^{-5} M FMLP, or 10^{-8} M PAF and the increase in chemiluminescence was followed for 20 min.

2.5. *Fluorescence studies*

Steady-state fluorescence anisotropy (r_s) measurements were performed at 37°C with a Perkin Elmer Spectrofluorimeter MPF-66

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00145793/90/$3.50

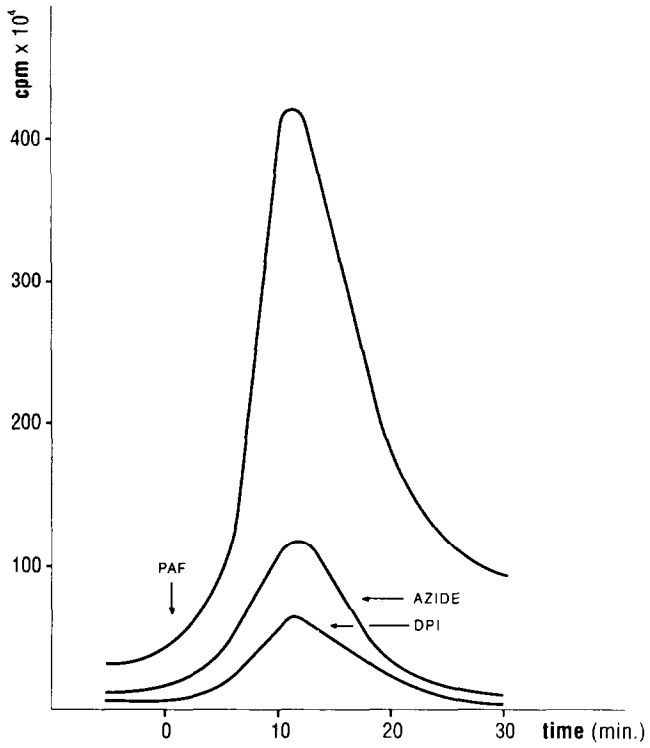

Fig. 1. Luminol amplified chemiluminescence of PMNs activated with PAF in the presence and absence of DPI and azide.

equipped with the Series 7000 Professional Computer for data acquisition, using TMA-DPH (Molecular Probes Inc., Eugene, OR, USA) as a hydrophobic fluorescent probe at a final concentration of 10^{-6} M as previously described [7]. After incubation with TMA-DPH for 5 min PMNs were stimulated by addition of stimulating agents (1.5×10^{-6} M PMA, 10^{-5} M FMLP, or 10^{-8} M PAF) in the absence and presence of inhibitors and the fluorescence anisotropy was measured for 20 min. Data were analysed by Student's *t*-test.

3. RESULTS

Luminol amplified chemiluminescence (CL) has been employed to verify that the superoxide-generating oxidase system in separated PMNs is dormant under resting conditions and can be activated by PMA, FMLP and PAF. Moreover, the effects of DPI (50 μM), azide (1 mM) and catalase (2000 U/ml) on the CL of resting and activated PMNs have been tested. Our data demonstrate a 90% inhibition of CL by DPI and a 85% inhibition by azide. No effect of catalase is observed. This is in accordance with our previous observations [14]. Fig. 1 shows the CL measurements of PMNs activated by PAF in the absence and presence of DPI and azide.

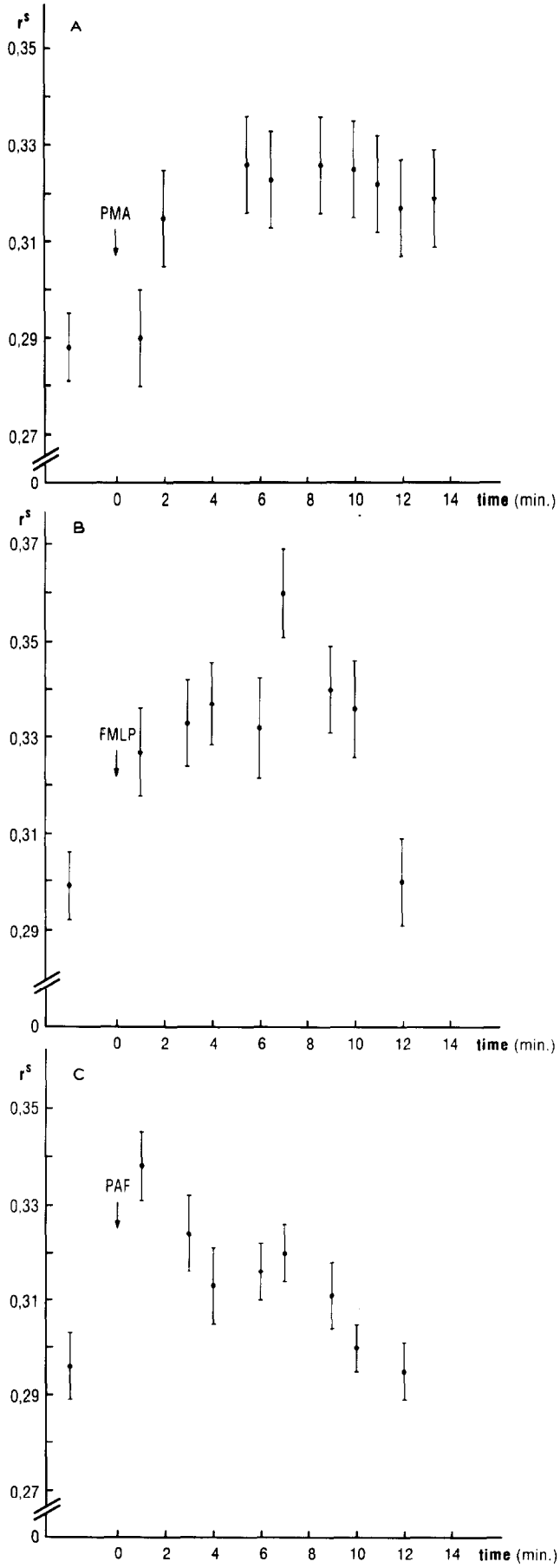

Fig. 2. Steady state fluorescence anisotropy (r_s) of TMA-DPH at 37°C in PMNs activated with PMA (A), FMLP (B), and PAF (C). Values are expressed as the mean ± SD of 20 samples. The *r* increase after activation is significant ($P < 0.05$).

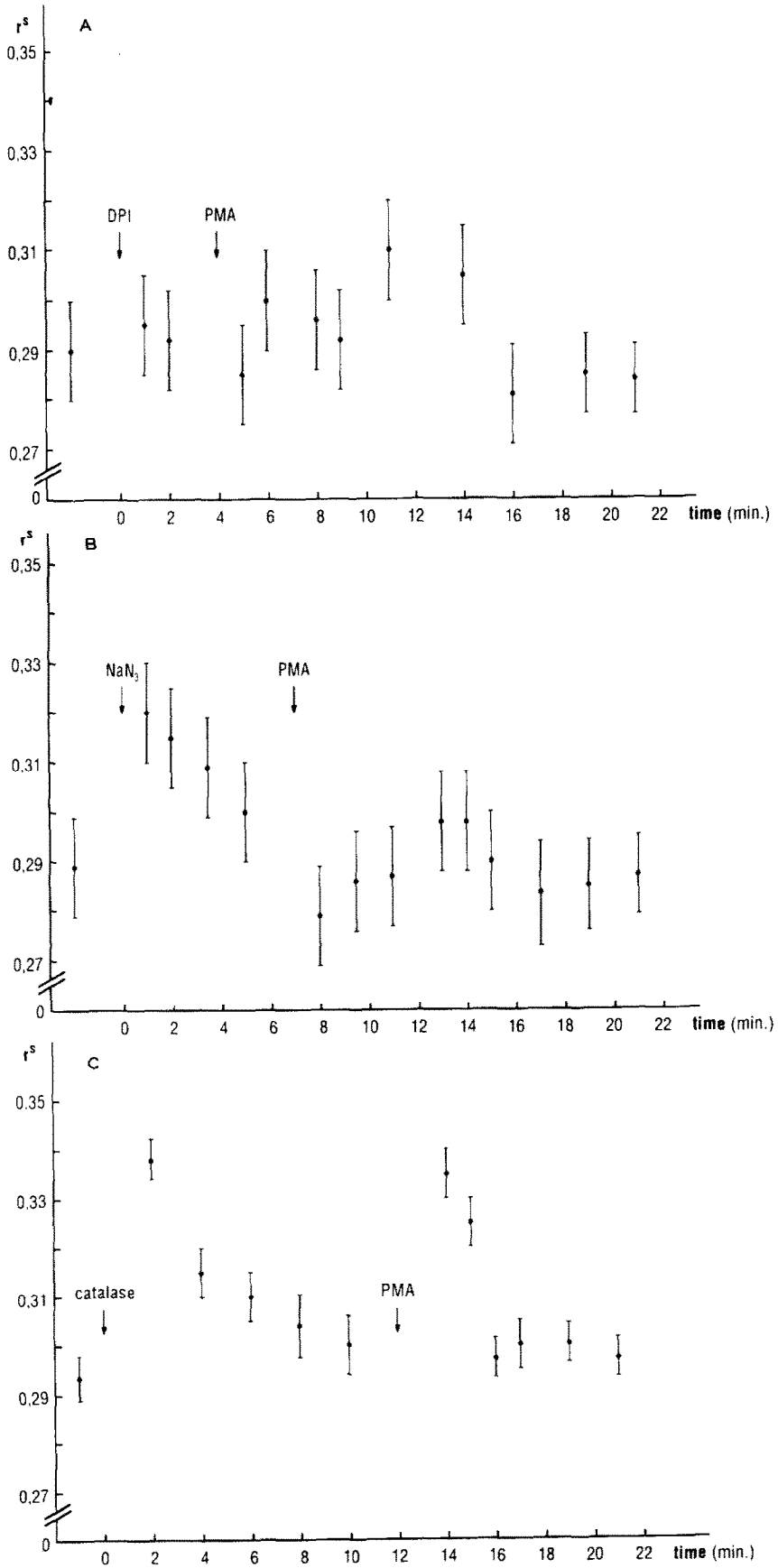

Fig. 3. Steady state fluorescence anisotropy (r_s) of TMA-DPH at 37°C in PMNs activated with PMA in the presence of DPI (A), azide (B), and catalase (C). Values are expressed as the mean ± SD of 10 samples.

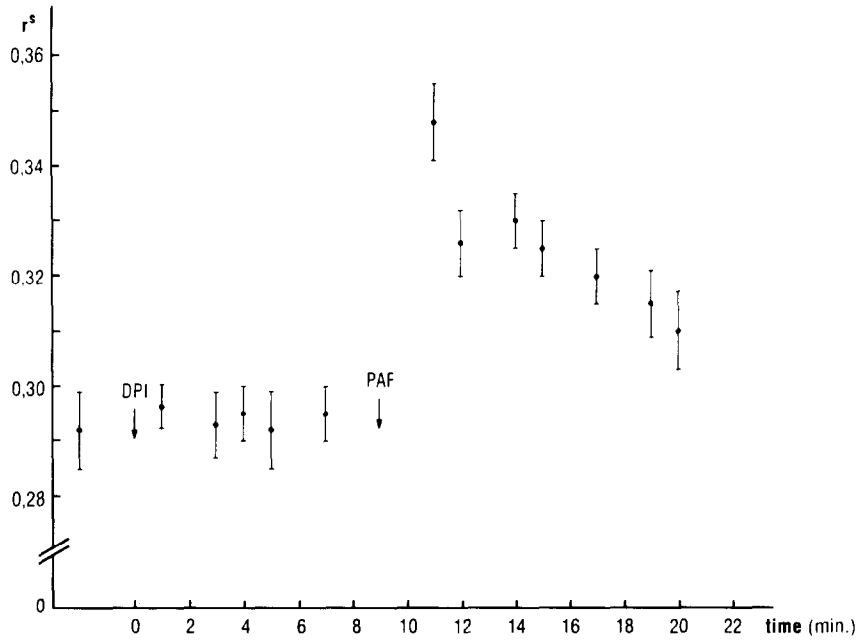

Fig. 4. Steady state fluorescence anisotropy (r_s) of TMA-DPH at 37°C in PMNs activated with PAF in the presence of PDI. Values are expressed as the mean ± SD of 10 samples.

Activation of resting PMNs by PMA (Fig. 2A), FMLP (Fig. 2B) and PAF (Fig. 2C) causes a significant increase ($P<0.05$) of TMA-DPH r_s. A lasting and stable increase in r_s value is observed 3 min after addition of PMA to resting PMNs. FMLP induces an increase in r_s value that reaches a peak value 7 min after activation, returning to the basal value after 12 min. A rapid increase in r_s value followed by a gradual decrease towards the basal value is observed after stimulation with PAF.

The r_s is not significantly changed after the addition of DPI to resting PMNs and after the consequent activation with PMA (Fig. 3A). However, using PAF as activator in the presence of DPI, a significant increase ($P<0.05$) of r_s is observed (Fig. 4). Azide induces a transient increase of r_s in resting PMNs. However, after activation with PMA no effect in r_s is observed (Fig. 3B). In the presence of catalase (2000 U/ml) a transient increase of r_s is observed in resting PMNs and following activation with PMA (Fig. 3C).

4. DISCUSSION

The RB in PMNs can be activated by a variety of agents. Regardless of the stimulus used, the activation pathways converge at the NADPH oxidase system [15]. This activation involves a number of biochemical events which occur at the level of the plasma membrane. In this study we have used as activators PMA, FMLP and PAF, which employ different stimulating pathways of oxidase activation and we have investigated the effect of these agents on the r_s of PMNs plasma membrane. TMA-DPH r_s of membranes reflects the packing of membrane lipid fatty acid chains and can be related to the order parameter S, if certain precautions are taken [16,17]. The lipid composition and therefore the packing can change upon certain stimuli and this has been shown to modulate cell function [18,19]. Such specific effects could be mediated through local lipid packing constraints which would affect the membrane protein conformation and the exposition of membrane components [19,20]. In concomitance with the RB activation we have observed physico-chemical changes of PMNs plasma membrane structure. The increase of r_s upon stimulation indicates an increase in order of membrane lipid domains and may be due to various processes accompanying the RB activation [2–6]. Since no changes of r_s occur upon activation of PMNs with PMA in the presence of DPI, which inhibits NADPH oxidase, it seems likely that the observed r_s changes are a consequence of NADPH oxidase activation.

PAF induces an increase of r_s of DPI-treated PMNs although the RB phenomenon is not activated as demonstrated by the chemiluminescence study. This is in accordance with recent observations of a non-specific effect of PAF on cell plasma membranes [21].

Recently a role of excited oxygen species, mainly H_2O_2, on the changes of the physico-chemical structure of PMNs plasma membrane has been proposed by Masuda et al. [10]. It is known that in the presence of azide, which inhibits myeloperoxidase, a supranormal release of H_2O_2 by activated PMNs occurs [22]. Since our results have shown no changes of r_s in azide treated PMNs after stimulation, it seems unlikely that the

above-reported increase of r_s upon activation of PMNs by the stimuli is mainly due to H_2O_2. To further analyze the role of H_2O_2 on r_s changes, we investigated the effect of catalase on PMNs activation. Addition of catalase induces a transient increase of r_s in resting PMNs and after stimulation with PMA. This supports the conclusion that H_2O_2 does not play a major role in r_s changes during the RB activation.

The increased order of the membrane lipid fatty acid chains accompanying RB activation, is not only related to the release of H_2O_2 but also to the various molecular events that occur during the RB. Further studies are in progress in our laboratory to explain and understand the mechanism which modulates the physico-chemical structure of the PMNs plasma membrane during activation.

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