Increased fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene in the phorbol myristate acetate-stimulated plasma membrane of human neutrophils

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1. INTRODUCTION

Phagocytosis is a key process in combatting infections in man and animals, and polymorphonuclear leucocytes (PMN) are of particular importance in this event. The plasma membrane of PMN plays a central part in phagocytosis. An important step in host defence by phagocytic PMN is an enhanced production of excited oxygen species [1] which takes place when resting cells are exposed to various activating agents such as bacteria and other particles, chemotactic peptides, the calcium ionophore A 23187 and certain complement components [2]. A particularly powerful activating agent is the tumor promoting phorbol-12-myristate-13-acetate (PMA) [3].

Several events, including generation of superoxide radicals ($O_2^{\cdot-}$), microtubule assembly, degranulation, enzyme secretion, release of arachidonic acid and its metabolites, and changes in cell membrane potential are initiated in the plasma membrane upon stimulation of resting PMN. Little is known about the exact sequence of these events and the mode of signal transfer between the stimulus and the cellular response. Here, we used the fluorescence anisotropy of the lipophilic probe diphenylhexatriene (DPH) to study possible physical changes of the PMN plasma membrane upon exposure to PMA. The results indicate a rapid and dramatic increase in the order of the membrane lipid domain upon exposure of the plasma membrane to this stimulus.

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Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; HBSS, Hank's buffered salt solution; PMA, phorbol-12-myristate-13-acetate; PMN, polymorphonuclear leucocytes

2. MATERIALS AND METHODS

Chemicals were obtained from the following sources: Fluka (Buchs), 1,6-diphenyl-1,3,5-hexatriene; Sigma (St Louis), 4β-phorbol 12β-myristate 13α-acetate; Pharmacia Fine Chemicals (Uppsala), Percoll, dextran T-500; Boehringer (Mannheim), cytochrome *c* (horse heart, salt free), nicotinamide adenine dinucleotide phosphate (reduced form, tetrasodium salt).

2.1. *Isolation of PMN*

Blood from healthy donors was collected and mixed with 6% dextran T-500 solution containing 0.9% of NaCl (5:1, v/v). After 30 min, the upper phase was removed and layered on a Percoll gradient and centrifuged for 20 min at 350 × *g* according to [4]. After centrifugation the granulocyte band (<97% PMN) was collected, and the cells were washed twice in HBSS (calcium free). The viability of the cells was determined by trypan blue exclusion and found to be ≥97%.

2.2. *Isolation of plasma membrane*

The procedure in [5] was followed except that the cells were ruptured by mild sonification on ice (3 × 10 s with the lowest energy output of a Branson type

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sonifier). Rat liver mitochondria and microsomes were prepared according to [6] and [7], respectively; the membrane of human red blood cells was isolated as in [8].

2.3. *Fluorescence polarization measurements*

Cells or the isolated membrane fractions (80–100 μg protein) were suspended in 3 ml HBSS and labeled for 20 min at 37°C with 2 μM DPH (2 mM stock solution in tetrahydrofuran). Steady state fluorescence anisotropy was measured with constant stirring in an Aminco SPF-500 spectrofluorometer. The sample was excited by a vertically polarized light at 357 nm, and the sample fluorescence at 430 nm was analyzed by using optical filters (cut off 420 nm) and the monochromator into vertically and horizontally polarized components I_V and I_H. The steady state anisotropy, r^s, is obtained as:

$$r^s = (I_V - I_H)/(I_V + 2I_H)$$

For each measurement of r^s, I_V and I_H were corrected for intrinsic fluorescence, light scattering and instrumental factors.

2.4. *Production of* $O_2^{\cdot-}$

The production of superoxide anion was continuously followed by the cytochrome *c* reduction assay [9].

2.5. *Challenge with PMA*

PMA (stock solution 2 mg/ml in dimethyl sulfoxide) was added to cells or membranes to give 1 μg or 330 ng/ml final conc. as indicated in section 3. The first determination of r^s of DPH was performed within 8 s after addition of PMA.

3. RESULTS

When resting intact PMN were stimulated with 1 μg PMA/ml, a small and rapid increase of the

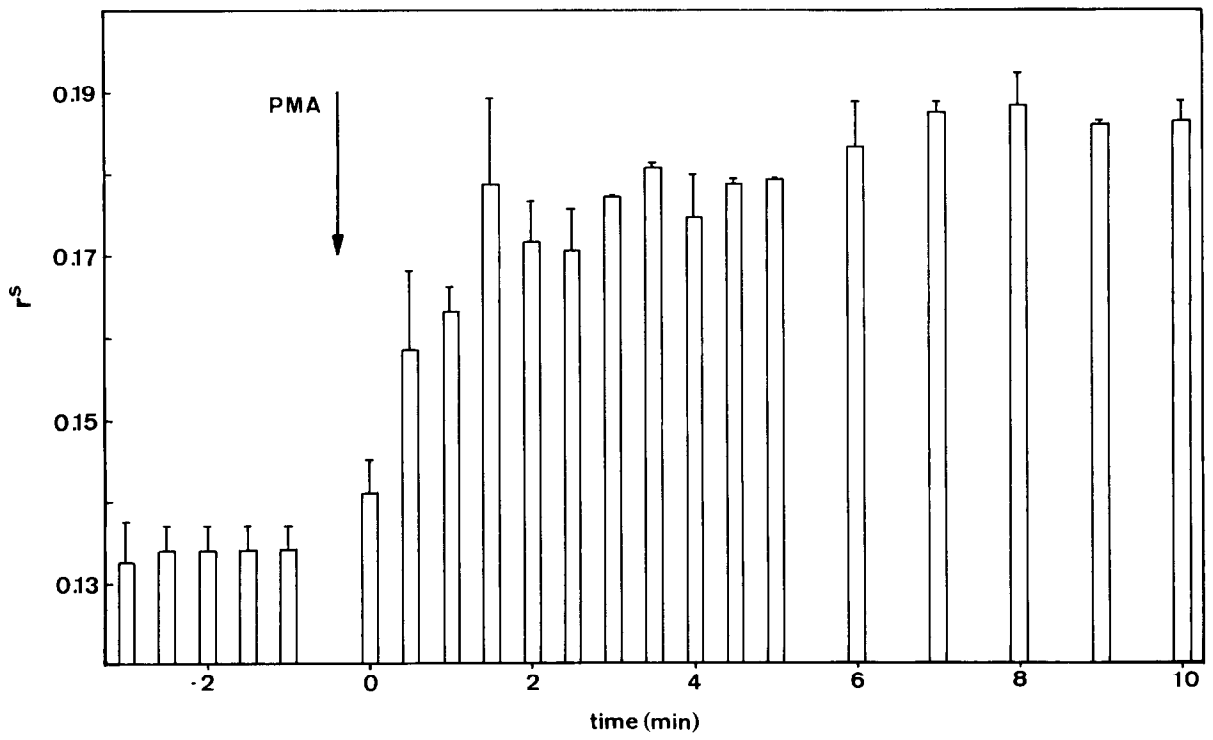

Fig.1. Steady-state fluorescence anisotropy (r^s) of DPH in the plasma membrane fraction of PMN upon stimulation by 1 μg PMA/ml. Cells were labeled with DPH for 20 min at 37°C in HBSS as in section 2 prior to the addition of PMA (indicated by the arrow). The result shown is representative of 3 separate experiments (± SD) each performed in duplicate.

fluorescence anisotropy (r^S) of DPH above a resting value of 0.18 was usually observed. It was essentially complete within 30 s. The increased r^S could be observed for ≥ 10 min. When smaller amounts (330 ng/ml) of PMA were used the increase of r^S was transient and could be evoked repetitively. The amount of $O_2^{\cdot-}$ produced by these cells upon stimulation, as measured by superoxide dismutase-inhibitable cytochrome *c* reduction, ranged from 50–70 nmol/min and mg protein.

DPH is known to incorporate spontaneously into all cellular membranes. The plasma membrane, which has been shown to contain receptors specific for PMA [10,11] constitutes, however, only a minor fraction of all membranes present in cells. Therefore the fluorescence polarization of DPH was also measured in the isolated plasma membrane fraction. Fig.1 shows that addition of PMA to the plasma membrane fraction isolated from resting PMN results in a rapid and very pronounced increase of r^S of DPH from 0.14–0.19. The fluorescence intensity, determined as $I_V + 2I_H$, of DPH increased ~1.8-fold upon PMA challenge. Identical results were obtained with the plasma membrane fraction isolated from PMN exposed to 1 μg PMA/10^7 cells for 3 min at 37°C prior to sonification of the cells (not shown). The increased r^S value in the stimulated plasma membrane fraction is lasting. Thus, when the stimulated plasma membrane fraction has been stored for 21 and 48 h, r^S was 0.18 and 0.17, respectively. The amount of cytochrome *c* reduced by the plasma membrane fraction in the presence of 1 mM NADPH was 7 nmol/min and mg protein, 30% of which were inhibitable by superoxide dismutase.

The specificity of the observed increase of r^S of DPH in the stimulated plasma membrane fraction is supported by the results shown in fig.2. PMA has no or only a minor influence on the fluorescence anisotropy of DPH imbedded in membranes of rat liver mitochondria or microsomes, or membranes of human red blood cells. No change of the fluorescence intensity of DPH upon addition of PMA was observed in these membranes.

The observed increase in r^S is not due to possible chemical reactions or degradation of DPH molecules upon stimulation of the plasma membrane fraction, as judged by the following control experiments: An r^S-value of 0.19 was also obtained when the isolated plasma membrane fraction was first stimulated with PMA and then labeled with DPH. Also, the shape of the fluorescence emission spectrum was the same regardless whether DPH was added before or after stimulation of the plasma membrane fraction by PMA (fig.3). The stimulation results in an increased fluorescence intensity.

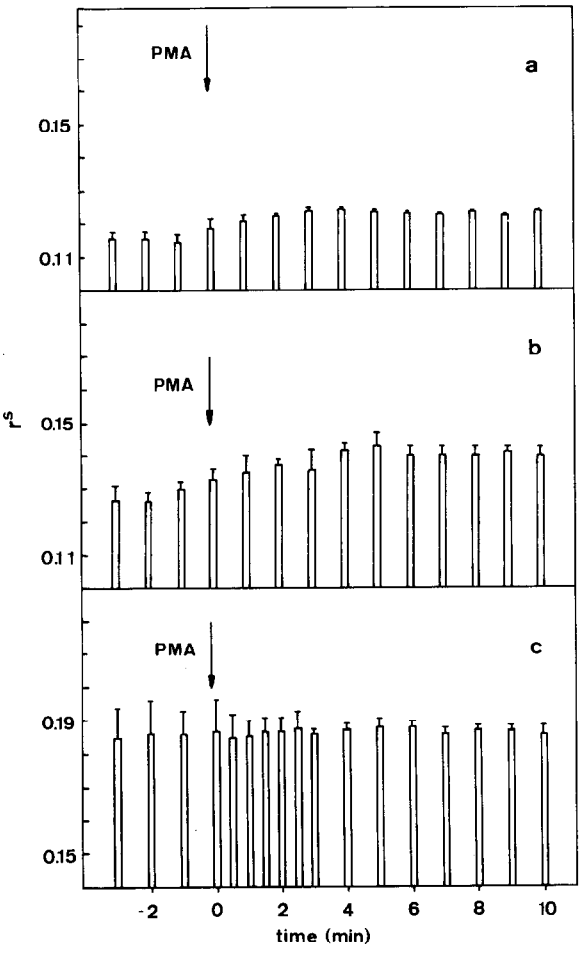

Fig.2. Steady-state fluorescence anisotropy (r^S) of DPH in rat liver microsomes (a), mitochondria (b), and membranes of human red blood cells (c) challenged with 1 μg PMA/ml. Measurements were performed as in fig.1. Each value represents the mean of 3 (a,b) and 4 (c) separate experiments (± SD).

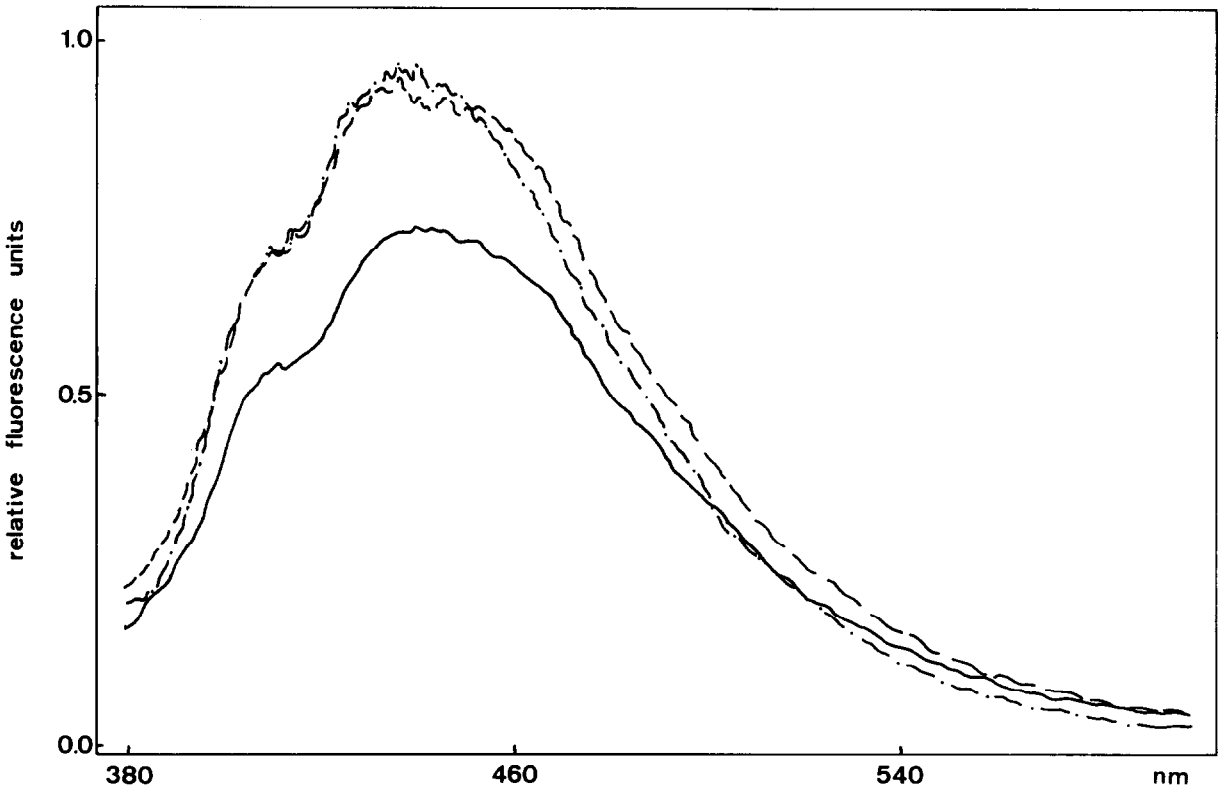

Fig.3. Fluorescence emission spectra of DPH in the plasma membrane fraction before (——) and after stimulation (- - -) by PMA. When adding DPH (2 μM) 5 min after stimulation of the plasma membrane fraction by PMA the emission spectrum (-·-·-) was recorded. In each experiment the plasma membrane fraction was labeled with DPH by an incubation for 20 min at 37°C. Excitation wavelength was 357 nm, the excitation and emission slits were 5 nm and 10 nm, respectively.

4. DISCUSSION

The steady state fluorescence polarization of DPH in membranes can be used as an indicator of the degree of order of membrane-lipid fatty acid chains, if certain precautions are taken [12–14]. Here, we show that stimulation by PMA increases the steady state fluorescence anisotropy, r^S, of DPH in intact human PMN. A dramatic increase of r^S is observed in the isolated plasma membrane fraction. The increase of r^S can result either from a decreased life-time of the excited DPH molecules, or an increase of the order of the fatty acid chains surrounding the DPH molecules. The former possibility can be excluded in our experiments by the observation that the fluorescence intensity of DPH increases upon stimulation, because the fluorescence life-time is approximately proportional to the fluorescence intensity [12]. Since significant changes in the r^S value of DPH in mitochondria, microsomes, and membranes of red blood cells are absent after stimulation by PMA its action appears specific for the plasma membrane of PMN. The results suggest that PMA causes a specific, rapid and extensive increase in the order of the lipid domain of the PMN plasma membrane. Definite proof of the increased order will be provided by time-resolved fluorescence polarization measurements.

After exposure of PMN with activating agents, the time required for the conversion from the resting to the fully stimulated state is between 20–70 s [1]. The observed increase of r^S is clearly detectable after 10 s, half-maximal after ~30 s and complete after ~2–3 min. The earliest events (e.g., changes

in membrane potential and calcium mobilization) observed so far also take place 5–10 s after stimulation [15,16]. The temporal sequences of these early responses have not been resolved so far.

The molecular events responsible for the increased order of the membrane lipid fatty acid chains after PMA stimulation are not known. The primary target of PMA in the plasma membrane is a protein receptor. It can be expected that the binding of the ligand to the receptor somehow transfers a signal to other proteins in the membrane (e.g., NADPH oxidase or a *b*-type cytochrome) involved in phagocytosis. Whether the transfer is mediated by an increase in the order of the lipid domain, or whether the observed increase is secondary to protein–protein interactions remains to be established.

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