Transmembrane potential variations accompanying the PMA-triggered O_2^- and H_2O_2 release by mouse peritoneal macrophages

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Stimulation by PMA of Streptococci-elicited macrophages induced a transient membrane depolarization preceding the onset of detectable O_2^- production. Mice-resident peritoneal macrophages were unresponsive to PMA for both activities. The PMA-triggered membrane depolarization seemed to be independent from O_2^- production because inhibition of membrane depolarization by EGTA had no effect on rates of O_2^- or H_2O_2 release and rate of antimycin A insensitive O_2 uptake by Streptococci-elicited macrophages. The portion of O_2 uptake recovered as O_2^- was found to be 1/3. The rate of O_2^- release was twice the rate of H_2O_2 production (1.1 nmol $H_2O_2 . min^{-1} . 10^6$ macrophages^{-1}).

Macrophage *Transmembrane potential* *Superoxide anion* *Hydrogen peroxide* *Extracellular Ca^{2+}*

1. INTRODUCTION

Macrophages activated for cytotoxicity appear to be able to release large amounts of hydrogen peroxide [1–3]. The molecular mechanism may be responsible, at least in part, of the tumoricidal activity shown by these activated macrophages [4–6]. The production of H_2O_2 is part of a more complex biochemical activation process referred to as 'respiratory burst' [7] beginning with the stimulation of a membrane-bound NAD(P)H oxidase, and resulting in a stimulation of the hexose monophosphate shunt, increased non-mitochondrial O_2 uptake and release of reduced oxygen metabolites (O_2^-, H_2O_2). Despite the putative involvement of active oxygen species in the macrophage cytotoxicity, attempts to detect a stimulation by neoplastic cells of the macrophage oxidative metabolism have been unsuccessful [3,8]. The triggering of the respiratory burst has been observed with membrane-acting stimuli (zymosan, polystyrene particles, ionophore A 23187, fatty acids, lectins, phorbol myristate acetate [9–11]. Some of the triggering agents, such as zymosan and PMA, have been widely used to study the oxidative metabolism of macrophage populations [3], but little is known about the early biochemical events preceding the stimulation of the respiratory burst.

To better understand the process of the respiratory burst stimulation by membrane perturbants, it would be useful to study a sensitive membrane parameter such as the transmembrane potential. In fact, the membrane potential of different types of cells has been shown to be sensitive to several stimulants [12–15] including PMA [16]. As to phagocytes, these investigations have been done mainly using PMN. Therefore, it would be of interest to develop this approach with macrophages, which can be modulated with respect to O_2^- production in close correlation with their state of acti-

Abbreviations: di-I-C1-(5), 3,3′-dimethylindodicarbocyanine iodide; DMSO, dimethylsulfoxide; EGTA, ethyleneglycol-bis (β-aminoethyl ether) *N,N*-tetraacetic acid; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; MEM, minimum essential medium; PDC, peritoneal exudate cells; PMA, phorbol myristate acetate; TPMP, triphenylmethyl phosphonium

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vation. Thus, we have investigated the effect of PMA on the transmembrane potential of macrophages able to produce large amounts of oxygen metabolites (H_2O_2, O_2^-). We have followed the transmembrane potential by using a fluorescent, positively-charged cyanine dye, sensitive to potential changes in cells [17,18]. It has been demonstrated that the fluorescence response of such dyes results from a potential-dependent partition of the dye between the cells and the extracellular medium, the accumulation of the dye leading to the formation of non-fluorescent dye aggregates inside the cell [19].

Here the time course of the potential variation was compared with the kinetics of the H_2O_2 and O_2^- production by two macrophage populations:

(i) Resident macrophages (i.e., macrophages from untreated mice) which are not activated for cytotoxicity and produce only small amounts of active oxygen species;

(ii) Macrophages elicited by a suspension of killed Streptococci. We have shown [20] that this population of phagocytes exhibited characteristics commonly attributed to inflammatory macrophages but display also properties of immunologically activated macrophages. We have observed a strong specific release of [^{3}H]thymidine or [^{3}H]proline from syngeneic prelabelled P815 mastocytoma cells co-cultivated with Streptococci-elicited macrophages. This tumoricidal activity is associated with an ability to release large amounts of oxygen metabolites [20].

2. MATERIALS AND METHODS

B6D2F$_1$(DBA/2 × C57/BL6) mice aged 7–8 weeks, obtained from CSEAL (CNRS-Orleans) were used.

Peritoneal exudate cells (PEC) were collected in MEM (Institut Pasteur Production) either from untreated mice or mice injected i.p. 4 days before with 1 ml of Streptococci suspension in saline [20] (concentration estimated by turbidimetry: $A_{540} = 4$). After centrifugation, the cells resuspended in RPMI 1640 medium (Gibco) supplemented with 5% FCS (Gibco) and antibiotics were adjusted to the appropriate macrophage concentration determined by neutral red uptake. If necessary 1 ml macrophage suspension was allowed to adhere on sterile 13 × 27 mm glass coverslips in 3.5 cm diam. Nunclon dishes (37°C, 5% CO_2 in air) for 2–4 h. Coverslips were rinsed twice with HBSS (Institut Pasteur Production) and kept in RPMI 1640 medium until required.

2.1. *Membrane potential measurements*

Membrane potential changes were monitored using the fluorescent cyanine dye di-I-Cl (5) ($\lambda_{ex} = 600$ nm, $\lambda_{em} = 650$ nm). Before the adherence period, macrophage concentration was adjusted to 7×10^6/ml. The coverslip was placed diagonally in a quartz cuvette of a JY-3 spectrofluorometer (Jobin-Yvon) with 0.04 μM di-I-Cl (5) in 3 ml HBSS stirred magnetically and maintained at 37°C. In some experiments EGTA (final conc. 2.5 mM) was added before PMA (Sigma; PMA 1 μM; DMSO 0.03%) TPMP 5 mM was used to abolish the transmembrane potential. Cyanine dye and TPMP were a generous gift from Dr L. Letellier.

2.2. *Superoxide anion production*

Macrophages (2×10^6) were plated in dishes containing coverslips. After adherence, the coverslip was introduced in a cuvette of a S67 spectrophotometer Leres (France). The O_2^- production, triggered by PMA (PMA, 0.1 μM; DMSO, 0.003%) was monitored at 550 nm using the SOD-inhibitable reduction of ferricytochrome *c* (Sigma, 53 μM) by O_2^- [21] ($\Delta\epsilon_M$ 21000) in 3 ml HBSS (37°C, stirred magnetically). In some experiments, 2.5 mM EGTA was added before PMA. Controls were done with SOD (Sigma) (50 U/ml).

2.3. *Assay for H_2O_2*

H_2O_2 release measurement was based on the oxidation of 2 molecules of ferrocytochrome *c* (21 μM) by 1 molecule of H_2O_2, catalysed by horseradish peroxidase (Sigma, 0.16 μM) and monitored at 550 nm ($\Delta\epsilon_M$ 21000) [22]. All other experimental conditions were as described in 'superoxide anion production' so that it was possible to monitor routinely the H_2O_2 release after the O_2^- production with the same macrophage-coated coverslip. This sample was transferred from a cuvette containing the appropriate medium for O_2^- production measurement to a cuvette with the preheated medium required for H_2O_2 assay.

2.4. *Assay for O_2 uptake*

The antimycin A (Sigma)-insensitive O_2 uptake triggered by PMA (PMA, 1 μM; DMSO, 0.03%) was measured polarographically with a Clark electrode, on a suspension of PEC, at 37°C (fig. 4). The contribution to this O_2 uptake of non-adherent peritoneal cells purified by plating 10×10^6 PEC for 4 h was negligible (not shown) and the antimycin A-insensitive O_2 consumption varied linearly with the macrophage concentration (fig. 5).

3. RESULTS

Addition of adherent macrophages to the assay medium was followed by a rapid decrease in the fluorescence signal. To investigate whether the fluorescence decrease was either mainly due to a membrane potential-dependent accumulation of the dye di-I-C1 (5) within the cells or simply caused by adsorption of the dye on cells and coverslip, we added TPMP. High concentrations of the lipophilic cation abolished the transmembrane potential [23]. The complete recovery of the initial fluorescence level following addition of TPMP indicated that the fluorescence decrease observed after introduction of macrophages in the cuvette was potential dependent (fig. 1).

The effect of PMA was investigated with two different macrophage populations: group C Streptococci-elicited macrophages, used as a source of macrophages activated for cytoxicity and resident macrophages. PMA was ineffective on resident macrophages but induced a transient depolarisation on Streptococci-elicited macrophages (fig. 1). The maximum amplitude of the depolarisation was reached 4 min after PMA addition, with a lag period of ~40 s (fig. 2).

O_2^- and H_2O_2 productions were measured on Streptococci-elicited macrophages using the same macrophage coverslip, in the same experimental conditions, within 0.5 h, so that extracellular release of H_2O_2 and O_2^- could be conveniently compared. As reported in (fig. 3), the rate of O_2^- release (1.1 nmol.min^{-1}.10^6 macrophages^{-1}) is about twice the rate of H_2O_2 release (0.56 nmol.min^{-1}.10^6 macophages^{-1}). These data support the widely accepted hypothesis of an O_2^- derived origin of H_2O_2, consecutive to the dismutation of O_2^- to O_2 and H_2O_2. But it is also possible that

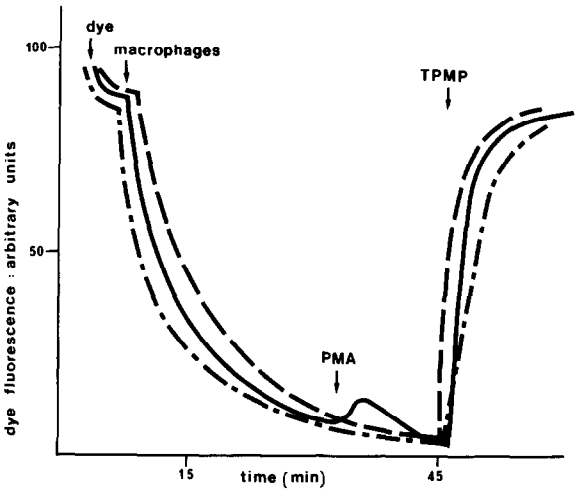

Fig. 1. Changes of di-I-C1-(5) fluorescence upon addition of macrophages, PMA and TPMP (representative experiment). Addition of macrophages is followed by a fluorescence decrease of the cyanine dye (0.04 μM). PMA induced a transient depolarisation on Streptococci-elicited macrophages (▬) whereas no detectable effect was observed either with resident macrophages (▪▬▪) or with Streptococci-elicited macrophages when 2.5 mM EGTA was added in the assay medium before the cyanine dye (▬▬). TPMP at 5 mM abolished the potential dependent fluorescence decrease.

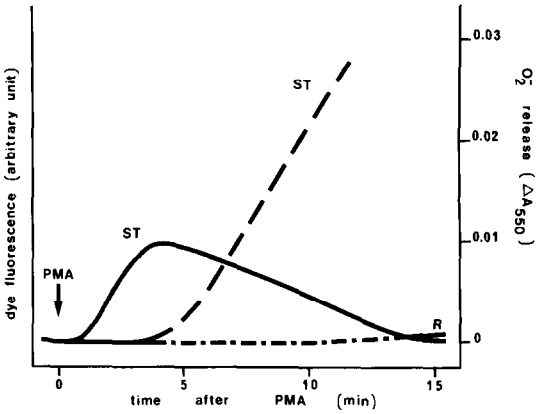

Fig. 2. Time-course of transmembrane potential variation and O_2^- release by macrophages after PMA triggering. Ordinate: Fluorescence of the dye (0.04 μM) using Streptococci-elicited macrophages (ST). Kinetic parameters (lag period, amplitude ...) have been calculated from 3 independent experiments (▬). Increase in ferricytochrome *c* absorbance (53 μM) after PMA addition: Streptococci-elicited macrophages (ST) (▬▬); resident macrophages (R) (▪▬▪). Kinetic parameters (lag period, rate value) are means of 3 independent experiments. Controls were done with SOD (50 U/ml).

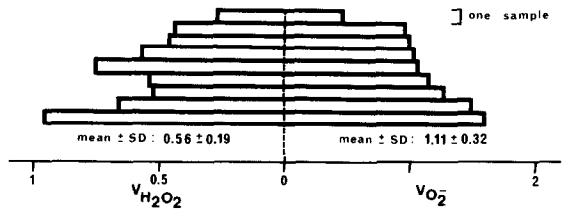

Fig. 3. Comparison between O_2^- and H_2O_2 production by Streptococci-elicited macrophages after PMA triggering. Both rates of O_2^- and H_2O_2 release have been determined on each sample (see section 2). Right panel: rate of O_2^- production expressed as nmol O_2^- . min^{-1} . 10^6 macrophages^{-1}. Controls were done with SOD (50 U/ml). Left panel: rates of H_2O_2 obtained with the corresponding samples, expressed as nmol H_2O_2 . min^{-1} . 10^6 macrophages^{-1}.

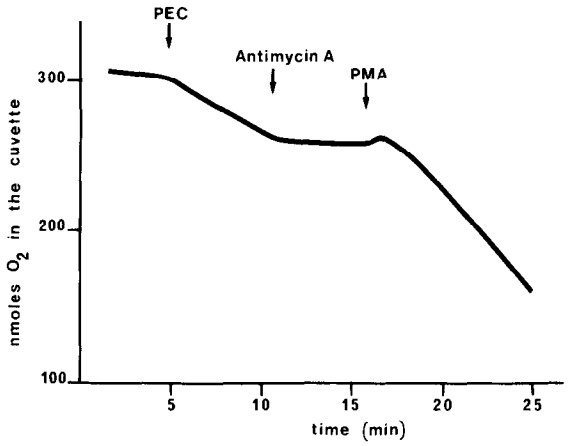

Fig. 4. Determination of antimycin A-insensitive O_2 consumption by Streptococci-elicited macrophages (representative experiment). The mitochondrial respiration of PEC was inhibited by antimycin A (0.22 μg/ml) before PMA addition (1 μM) triggered the macrophage respiratory burst.

H_2O_2 extracellular release occurred independently from that of O_2^-. The rate of antimycin A-insensitive O_2 consumption measured on a suspension of peritoneal exudate cells (fig. 4) was ~3-fold the rate of O_2^- release (3 nmol . min^{-1} . 10^6 macrophages^{-1}). This O_2 uptake was not due to non-adherent PEC: the antimycin A-independent O_2 consumption was found to vary linearly with the macrophage concentration (estimated by neutral red uptake), although the ratio of macrophage to

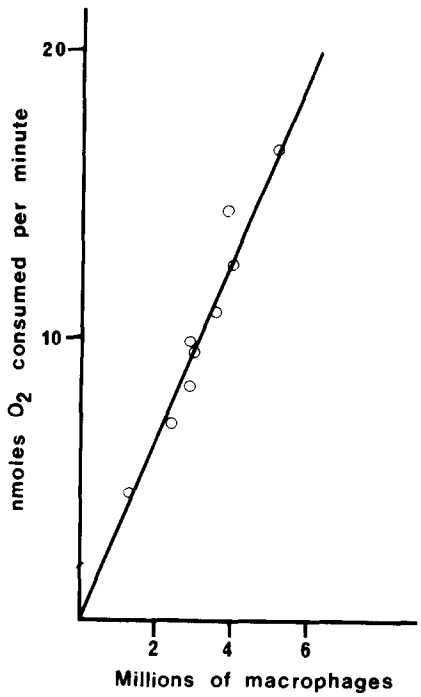

Fig. 5. Rate of antimycin A-insensitive O_2 uptake by Streptococci-elicited macrophages. Abscissa: total number of macrophages present in the sample, determined by neutral red uptake. Ordinate: rate of non-mitochondrial O_2 uptake, determined as in fig. 4.

non-adherent cells was different from one experiment to another (fig. 5). Direct measurements of antimycin A-inhibited O_2 uptake by non-adherent PEC alone have confirmed this result (not shown).

The time course of the macrophage membrane depolarisation was compared with the first minutes of the O_2^- production by Streptococci-elicited macrophages (fig. 2). With a lag period of 3 min, the O_2^- release began after maximal membrane depolarisation during the repolarisation period. Despite a possible difference in the sensitivity of the 2 methods employed, this observation supported the hypothesis of sequential effects of PMA on the macrophage membrane. This hypothesis of a primary membrane depolarisation requirement for PMA stimulation of the respiratory burst was also supported by the following correlation. Resident macrophages, which could not respond to PMA by membrane depolarisation were also unable to produce increased amounts of reduced oxygen species after PMA triggering (fig. 2).

Table 1

Influence of extracellular Ca^{2+} on the oxidative burst

No. exp.	Addition to the assay medium	O_2[a]	O_2^-[b]	H_2O_2[c]
1	None	3.8	1.6	0.95
	EGTA	3.5	2.02	0.85
2	None	3.1	0.97	0.45
	EGTA	3.2	0.97	0.46

[a] O_2 consumption measured as in fig. 4

[b] O_2^- release measured by reduction of ferricytochrome *c* (53 μM) in 3 ml HBSS; controls were done with SOD (50 U/ml)

[c] H_2O_2 release measured by ferrocytochrome *c* oxidation (final conc. 21 μM in 3 ml HBSS) catalysed by 0.16 μM horseradish peroxidase

EGTA (2.5 mM) was added 10 min before PMA, used to stimulate O_2^- or H_2O_2 release and O_2 uptake by Streptococci-elicited macrophages. Two independent experiments. Values are expressed as nmol metabolites $.min^{-1}.10^6$ macrophages^{-1}

We therefore investigated the effect of EGTA on PMA-dependent macrophage membrane depolarisation and O_2^- production. The latter parameter was insensitive to EGTA (table 1) but the former was entirely inhibited by excess of Ca^{2+} chelator agent (fig. 1). To check the influence of extracellular Ca^{2+} on the respiratory burst we measured both the non-mitochondrial O_2 consumption and the H_2O_2 released by Streptococci-elicited macrophages after PMA triggering, with EGTA added in the assay medium. Antimycin A-insensitive O_2 uptake and H_2O_2 production were also found to be insensitive to EGTA (table 1).

4. DISCUSSION

Stimulation of macrophages by phorbol myristate acetate has been shown to induce variations of numerous biochemical parameters. PMA has been reported to increase plasminogen activator synthesis and secretion [24,25], to increase prostaglandin and arachidonic acid metabolism [26], to produce a selective release of lysosomal acid hydrolases [26] and to trigger metabolic changes usually referred to as the respiratory burst of phagocytes [3]. All these modifications are the final expression of some PMA effects on macrophages. However, little is known about the early biochemical events following the interaction of PMA with macrophages.

We reported here that one of the early changes is a reversible membrane depolarisation, observed with a particular macrophage population. This PMA-mediated depolarisation was correlated with a concomitant stimulation of the respiratory burst (i.e., increased O_2 uptake and O_2^- or H_2O_2 production). Resident macrophages failed to show either membrane depolarisation or oxygen metabolites production. In contrast, Streptococci-elicited macrophages responded to PMA by a membrane depolarisation and H_2O_2 or O_2^- production. The transient depolarisation always preceded production of oxygen species. Similar observations have been obtained by J.C. Within, with human granulocyte suspensions [16].

Using EGTA, we have differentiated two PMA effects by their dependence on extracellular Ca^{2+}. Membrane depolarisation was inhibited by EGTA, suggesting an involvement of extracellular Ca^{2+} in the membrane potential variation. A Ca^{2+} influx from the external medium into the macrophage intracellular compartment might explain the Ca^{2+} dependence of the depolarisation. This hypothesis would be in good agreement with the Ca^{2+}-related increase in intracellular cAMP level reported [25] with oil-elicited guinea pig macrophages, after addition of phorbol myristate acetate.

No effect of EGTA could be observed on the respiratory burst: neither O_2 consumption nor O_2^- and H_2O_2 production by Streptococci-elicited macrophages was inhibited by EGTA. Similar results have been obtained using intact PMN [28,29].

These results suggest that the depolarisation induced by PMA is not the membrane event triggering the respiratory burst. Addition of TPMP resulting in partial or total membrane depolarisation did not stimulate the extracellular O_2^- release (not shown). So, it seems that membrane depolarisation and stimulation of the respiratory burst which can be simultaneously triggered by the same agent (PMA), are independent phenomena. The experiments performed with EGTA confirmed this view. Whereas the transient membrane depolarisation is abolished when extracellular Ca^{2+} is close to

0.2 μM (calculated using a chelation constant of $\sim 5 \times 10^6$ M^{-1}) [30], the integrity of the respiratory functions is preserved.

The discrepancy observed between the rate of non-mitochondrial O_2 consumption measured on cell suspensions, and the rate of O_2^- and H_2O_2 release, measured on adherent macrophages could be another illustration of differences between macrophages monolayers and suspensions. However, such a disparity was reported when phagocyte suspensions are used for both assays. The portion of O_2 reduced to extracellular O_2^- and H_2O_2 varied with the cell type [7] the trigger used [31] and represented from 17–100% of the total O_2 uptake [7,31]. Concerning the fate of the O_2 excess, one explanation could be that the release of oxygen-derived metabolites in the extracellular medium is accompanied by an intracellular production of O_2^- and H_2O_2. Within the phagocytes, at least part of these oxygen species is enzymatically reduced by catalase, peroxidase or via glutathione cycle [7] and cannot diffuse outside the cell where it would be detected by classic ferricytochrome *c* oxidation.

Furthermore, to explain the difference between O_2 uptake and O_2^- or H_2O_2 release, we have to consider that PMA is able to trigger not only the respiratory burst but also some other metabolic pathways, including prostaglandin synthesis [26,32]. And it is well known that the first step of this synthesis is an enzymic oxidation of arachidonic acid by molecular oxygen. Streptococci-elicited macrophages effectively released PGE_2 in response to PMA triggering although this activity was less than in resident peritoneal macrophages [20].

Thus, it appears that a certain part of the O_2 consumed during the respiratory burst, is in fact involved in biochemical processes requiring molecular oxygen as oxidating factor (e.g., prostaglandin synthesis).

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