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A time-course study on superoxide generation and protein kinase C activation in human neutrophils

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The time course of superoxide generation and membrane association of protein kinase C was studied in human neutrophils stimulated by PMA, FMLP, ionomycin and A23187. The initiation of superoxide generation in PMA; ionomycin- and A23187-stimulated neutrophils was characterized by a lag period of at least 30 s in contrast to a lag period of 10–15 s in FMLP-stimulated cells. The time course of membrane association of protein kinase C in PMA-stimulated neutrophils was highly dependent upon the PMA concentration used for stimulation. However, membrane association of protein kinase C preceded superoxide generation in cells stimulated by 10–300 ng/ml PMA. FMLP, ionomycin and A23187 induced membrane association of protein kinase C in a few seconds and always before superoxide generation. It is concluded that membrane association of protein kinase C in PMA-, FMLP-, ionomycin- and A23187-stimulated neutrophils precedes superoxide generation, and thereby may be part of the mechanism initiating NADPH-oxidase activity. A simple correlation between the two parameters could not be proven, indicating that also other activation mechanisms are decisive in the activation of NADPH-oxidase.

Superoxide; Protein kinase C; Phorbol ester; Leukocyte; Membrane association

1. INTRODUCTION

Production of free radicals is an essential part of the response observed in neutrophils upon phagocytosis [1]. Superoxide ions, produced by NADPH-oxidase, constitute one of the main toxic free radicals and many studies have focused on the mechanism responsible for activation of NADPH-oxidase [2,3]. The calcium- and phospholipid-dependent protein kinase, protein kinase C, is hypothesized to be one of the key enzymes in activation of NADPH-oxidase [4]. In vivo receptor-induced activation of protein kinase C is proposed to involve a GTP-binding regulatory protein (G-protein), which stimulates phospholipase C leading to phosphoinositol hydrolysis [3,5]. The subsequent production of diacylglycerol leads to an association of protein kinase C with the cell membrane and thereby activation of the enzyme [3,6]. Many stimuli leading to superoxide generation in neutrophils are observed to induce a membrane association of protein kinase C [7] and it is proposed that protein kinase C activation is decisive in NADPH-oxidase stimulation [3]. However, this hypothesis requires that protein kinase C activation and thereby its membrane association precedes superoxide generation. Therefore, the time course of superoxide generation and membrane association of protein kinase C was investigated in human neutrophils.

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine

2. MATERIALS AND METHODS

2.1. *Materials*

Cytochalasin B and ionomycin were obtained from Sigma

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Chemical Company (St. Louis, MO, USA). Other materials were obtained as described [8–10].

2.2. *Buffer solutions*

Buffer A is 20 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, 500 μg/ml soybean trypsin inhibitor, 1 mM benzamidine, and 1 mM dithiothreitol. Buffer B is 20 mM Tris-HCl (pH 7.5), 10 mM EGTA, 4 mM EDTA, 500 μg/ml soybean trypsin inhibitor, 1 mM benzamidine, and 1 mM dithiothreitol. Buffer C is 20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 2 mM EDTA, 20 μg/ml soybean trypsin inhibitor, 1 mM benzamidine, and 1 mM dithiothreitol. Buffer D is as A but with 20 μg/ml soybean trypsin inhibitor.

2.3. *Purification of neutrophils*

Neutrophils were purified as described [11] except that the cells were finally washed twice in Krebs-Ringer phosphate buffer.

2.4. *Assay for superoxide generation*

Superoxide generation was assayed as reduction of cytochrome *c*. 2×10^6 neutrophils were preincubated for 5 min in 1 ml Krebs-Ringer phosphate buffer containing 5 mM glucose and 1 mg/ml cytochrome *c*. Cells activated by FMLP or A23187 were further incubated for 5 min in the presence of 10 μg/ml cytochalasin B and the cells were subsequently activated by PMA, FMLP, ionomycin or A23187 as indicated in the legends to the figures. Reduction in cytochrome *c* was continuously measured as change in absorbance at 550 nm.

2.5. *Assay for translocation of protein kinase C activity*

3×10^6 human polymorphonuclear leukocytes were preincubated for 10 min at 37°C in 1.5 ml Krebs-Ringer phosphate buffer containing 5 mM glucose and cells to be stimulated with FMLP were further incubated for 5 min in the presence of cytochalasin B (10 μg/ml). The cells were then incubated with PMA, FMLP, ionomycin or A23187 for the time indicated in legends to figures. The incubation was terminated by a 5 s centrifugation at 9000 × *g*, addition of 1 ml buffer A and immediate sonication. The crude homogenate was centrifuged at 50000 × *g* for 60 min, the pellet (particulate fraction) was resuspended in 1.5 ml buffer B containing 1% Triton X-100, stored on ice for 60 min, and centrifuged at 50000 × *g* for 30 min. The solubilized particulate fractions were chromatographed in parallel on DEAE cellulose columns (0.2 × 1 cm) equilibrated with buffer C. The columns were washed with 5 ml buffer C and 2 ml buffer D. Protein kinase C was eluted with 0.5 ml buffer D containing 100 mM NaCl and the columns were further washed with 2 ml of the same buffer. Phospholipid-independent protein kinase activity was then eluted by 0.5 ml buffer D containing 400 mM NaCl. Protein kinase C activity was assayed in the presence or absence of phosphatidylserine and diolein as described [8,9].

3. RESULTS AND DISCUSSION

The time course of superoxide generation and membrane association of protein kinase C was studied in human neutrophils. The neutrophils were stimulated by PMA (which substitutes for diacylglycerol and stimulates protein kinase C directly), FMLP (which is believed to activate phospholipase C and thereby give rise to increased intracellular calcium and protein kinase C activation), A23187 and ionomycin (calcium ionophores). These stimulators were selected because they are widely used and represent three different ways of protein kinase C activation. As the cell concentration is known to be essential to both membrane association of protein kinase C and superoxide generation, these parameters were measured in parallel experiments using equivalent cell concentrations. Also a previously published assay [10] used to measure membrane associated protein kinase C activity was optimized and this allowed the determination of membrane-associated protein kinase C after a few seconds stimulation of 3×10^6 neutrophils.

In PMA-treated cells, the time course of both membrane association of protein kinase C and superoxide generation was dependent upon the PMA concentration used for stimulation (fig.1). Superoxide generation in PMA-stimulated neutrophils was characterized by a lag period of at least 1 min depending upon the PMA concentration. However, membrane association of protein kinase C was observed before superoxide generation independently of the used PMA concentration (fig.1A–C).

In FMLP-stimulated neutrophils membrane association of protein kinase C was observed only in cytochalasin B-treated cells. Maximal effect was obtained after incubation with 10 μg/ml cytochalasin B and 300 nM FMLP (not shown). The membrane association was detectable in a few seconds and maximal after 10–30 s and then declined (fig.2A). Superoxide generation was induced after a lag period of 15–20 s and was maximal after approx. 1 min (fig.2A).

Ionomycin and A23187 induced superoxide generation in human neutrophils after a lag period of 30–60 s (fig.2B,C), while membrane association of protein kinase C was induced in a few seconds and always preceded superoxide generation.

Activation of NADPH-oxidase has been proposed to involve a direct phosphorylation of the enzyme by protein kinase C [12]. If membrane association of protein kinase C is decisive for this phosphorylation and thereby activation of

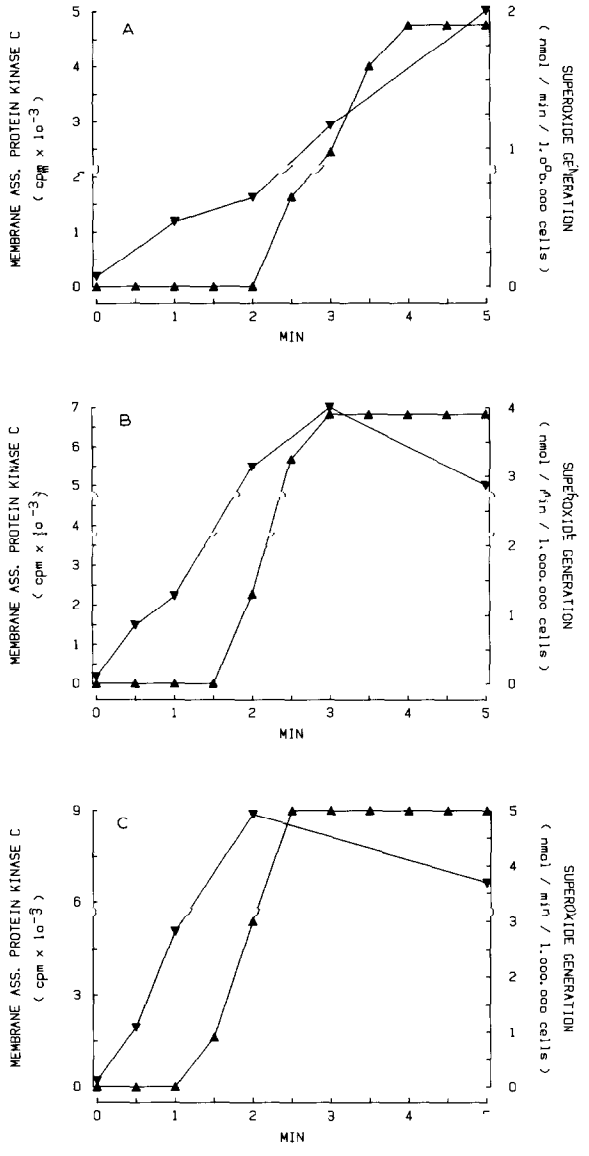

Fig.1. Superoxide generation (▲) and membrane associated protein kinase C (▼) were measured in human neutrophils stimulated by PMA (A, 10 ng/ml; B, 30 ng/ml; C, 300 ng/ml) for the time indicated. The figures are representative of three performed experiments.

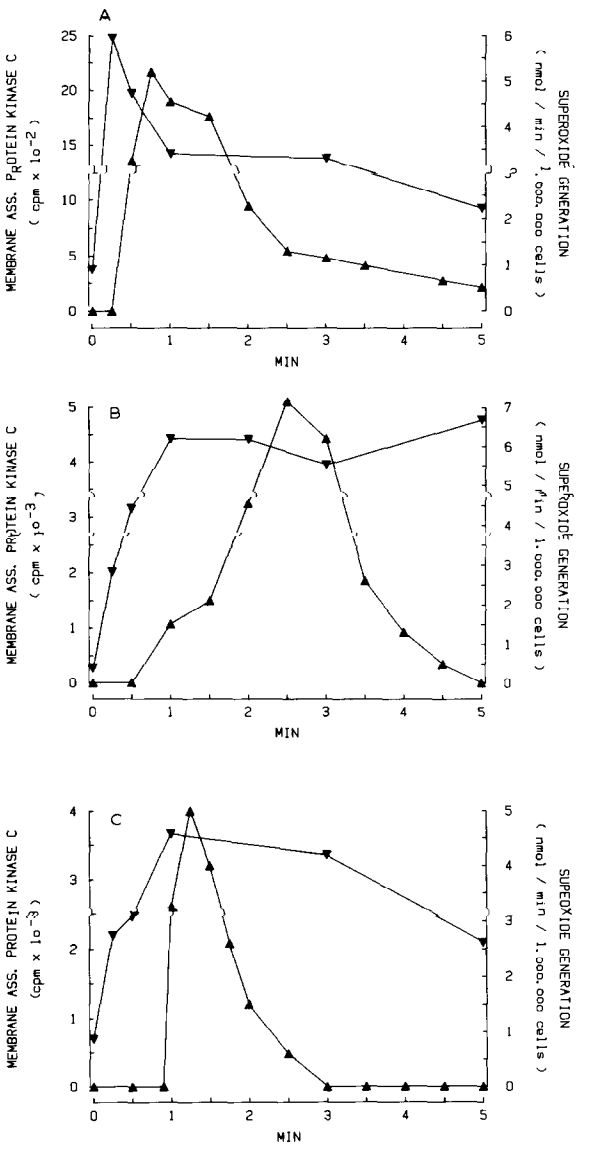

Fig.2. Superoxide generation (▲) and membrane associated protein kinase C (▼) were measured in human neutrophils stimulated by 1 μM FMLP (A), 3 μM ionomycin (B) and 3 μg/ml A23187 (C) for the time indicated. The figures are representative of three performed experiments.

NADPH-oxidase it has to precede the production of superoxide ions. Using PMA, FMLP, ionomycin and A23187 as activators, membrane association of protein kinase C in all experiments preceded superoxide generation. Therefore, we conclude that protein kinase C was active in superoxide producing neutrophils stimulated by PMA, FMLP, ionomycin and A23187. However, no direct correlation between protein kinase C activation and superoxide generation was detectable. In fact, the lag period between protein kinase C translocation and superoxide generation was only

a few seconds in FMLP-stimulated cells (fig.2A) in contrast to at least 30 s in PMA-, ionomycin- and A23187-stimulated neutrophils. Also a decline in superoxide generation in A23187- and ionomycin-stimulated cells was observed after 1–2 min, although an equivalent decrease in protein kinase C translocation was not detectable (fig.2B,C). This indicates that mechanisms other than protein kinase C activation are involved in the activation of NADPH-oxidase. A change in cytosolic free calcium has been speculated to constitute an additional signal in the activation of NADPH-oxidase. However, both the membrane-associated protein kinase C activity and intracellular calcium are highly elevated in ionomycin- and A23187-stimulated cells, the lag period between protein kinase C translocation and superoxide generation is still not comparable to that observed in FMLP-stimulated cells. This indicates that a further signal other than protein kinase C and intracellular calcium is involved in the activation of NADPH-oxidase. A similar conclusion was reached by Grinstein and Furuya [13] using electrically permeabilized neutrophils and the protein kinase C inhibitor H7.

In conclusion, the results indicate that protein kinase C activation precedes superoxide generation in PMA-, FMLP-, ionomycin- and A23187-stimulated neutrophils. However, the lag period between protein kinase C activation and superoxide generation differs depending on the stimulus used indicating that other activation mechanisms may also be decisive in the activation of NADPH-oxidase.

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