Guanine nucleotides stimulate NADPH oxidase in membranes of human neutrophils

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In the chain of events by which chemotactic peptides stimulate NADPH oxidase-catalyzed superoxide formation in human neutrophils, the involvements of a pertussis toxin-sensitive guanine nucleotide-binding protein (N-protein), mobilization of intracellular calcium and protein kinase C stimulation have been proposed. Superoxide formation was studied in membranes from human neutrophils; NADPH oxidase was stimulated by arachidonic acid in the presence of neutrophil cytosol. Fluoride and stable GTP analogues, such as GTPγS and GppNHp, which all activate N-proteins, enhanced NADPH oxidase activity up to 4-fold. GDPβS inhibited the effect of GTPγS. These data suggest that NADPH oxidase is regulated by an N-protein, independent of an elevation of the cytoplasmic calcium concentration.

Neutrophil *NADPH oxidase* *Guanine nucleotide-binding protein* *Arachidonic acid*

1. INTRODUCTION

Binding of the chemotactic peptide, FMLP, to neutrophil membrane receptors induces several cellular responses including superoxide formation [1], release of arachidonic acid, phospholipase C-catalyzed degradation of phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate as well as mobilization of intracellular calcium [2,3]. All these FMLP-induced events involve a pertussis toxin-sensitive N-protein [2–4]. As PMA, which is an activator of protein kinase C, stimulates NADPH oxidase in intact neutrophils [1], it is widely assumed that activation of NADPH oxidase is mediated by protein kinase C-catalyzed phosphorylation of the oxidase or a regulatory component [1,5]. There is recent evidence indicating that NADPH oxidase activation by FMLP may not necessarily be linked to phosphoinositide turnover or activation of protein kinase C, as a transient increase in the intracellular calcium concentration is not sufficient for FMLP-induced NADPH oxidase activation [6]. In addition, inhibitors of protein kinase C block PMA- but not FMLP-induced superoxide formation [7,8]. Furthermore, in calcium-depleted human neutrophils primed with subthreshold concentrations of PMA, FMLP activates NADPH oxidase in the absence of phosphoinositide hydrolysis [9].

We studied the regulation of NADPH oxidase in membranes from unstimulated human neutrophils. These investigations were prompted by the finding that arachidonic acid activates superoxide formation in a cell-free system consisting of the particulate 48 000 × *g* fraction and the cytosolic fraction from human neutrophils [10]. We report here that stable GTP analogues

Abbreviations: AppNHp, adenylyl imidodiphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); FMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); GppNHp, guanylyl imidodiphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); N-protein, guanine nucleotide-binding protein; PMA, phorbol 12-myristate 13-acetate

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stimulate NADPH oxidase activity, suggesting the direct involvement of an N-protein in the regulation of this enzyme.

2. MATERIALS AND METHODS

Human neutrophils were isolated by dextran sedimentation and centrifugation through Ficoll-Hypaque (Biochrom, Berlin) and disrupted by nitrogen cavitation. Neutrophil membranes and cytosol were isolated by centrifugation on discontinuous Percoll gradients (Pharmacia, Uppsala) [11], harvested and stored at −80°C.

NADPH oxidase activity of neutrophil membranes was determined by measuring the linear rate of superoxide dismutase-inhibitable ferricytochrome *c* reduction at 27.5°C [10]. The assay mixture (500 μl) contained 2–7 μg neutrophil membrane protein, 30–170 μg cytosolic protein, 500 μM NADPH, 10 μM FAD, 100 μM ferricytochrome *c*, 3.4 mM $MgCl_2$, 200 μM ATP, 500 μM EGTA and 50 mM triethanolamine-HCl, pH 7.0. Although the specific activity of NADPH oxidase varied to some extent between membrane preparations as observed by Jones et al. [12], qualitatively comparable results were obtained in all preparations analyzed.

For pertussis toxin treatment of neutrophils, purified neutrophils were suspended at 8×10^7 cells/ml in carbogen-saturated buffer (138 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.1 mM $CaCl_2$, 0.1 mM EDTA, 1 mM Na_2HPO_4, 5 mM $NaHCO_3$, 5.5 mM glucose, and 20 mM Hepes, pH 7.4) and incubated for 3 h at 37°C with pertussis toxin (1 μg/ml) or its carrier. Cells were then washed twice in the same buffer, and membranes were isolated.

Protein determination was performed according to Lowry et al. [13]. NADPH, FAD, guanine and adenine nucleotides were from Boehringer Mannheim (Mannheim, FRG). Ferricytochrome *c* (Type III), superoxide dismutase and arachidonic acid (grade I, 99% pure, from porcine liver) were obtained from Sigma (Taufkirchen, FRG). A stock solution of 16 mM arachidonic acid in 100% ethanol was prepared under nitrogen and stored at −20°C.

3. RESULTS

Original registrations of superoxide formation are shown in fig.1. Arachidonic acid (16 μM) stimulated superoxide generation in neutrophil membranes in the presence of neutrophil cytosol; there was a lag time of 1–4 min from addition of arachidonic acid to reaching a constant rate of superoxide formation, depending on the membrane preparation used. In the absence of neutrophil cytosol, NADPH oxidase was not activated by arachidonic acid. Addition of the stable GTP analogue, GTPγS (10 μM), which activates N-proteins [14], 2 min prior to or with arachidonic acid enhanced the rate of superoxide production about 3-fold.

This effect was specific for non-hydrolyzable GTP analogues (fig.2). GTPγs and GppNHp stimulated superoxide formation about 4-fold; the effect of GTPγS was half-maximal at 1 μM, GppNHp was 2 orders of magnitude less potent. GTP, ATPγS and AppNHp at up to 300 μM had no effect. GDPβS did not stimulate NADPH oxidase activity but competitively inhibited the stimulatory effect of GTPγS (fig.3): 10 μM GDPβS caused half-maximal inhibition of the superoxide formation observed in the presence of 1 μM GTPγS. Fluoride, which is another potent activator of N-proteins [14], activates superoxide generation [15] and calcium mobilization [16] in intact neutrophils. In the presence of aluminium (5 μM $Al_2(SO_4)_3$), which is necessary for fluoride activation of N-proteins [17], fluoride (10 mM) enhanced NADPH oxidase activity about 4-fold, an effect comparable to that obtained with the

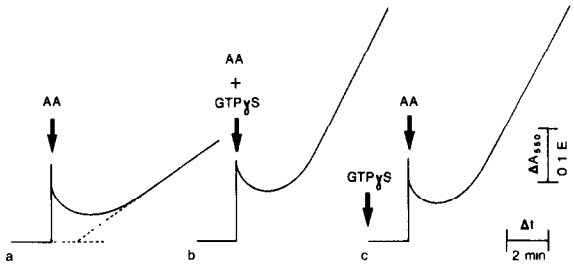

Fig.1. Time courses of superoxide generation in human neutrophil membranes. Superoxide formation was determined with 2 μg membrane protein in the presence of 30 μg neutrophil cytosol. The intersection of the dashed lines represents the lag time required for NADPH oxidase activation, defined from extrapolation of the absorbance curve to zero. AA indicates addition of arachidonic acid (16 μM), GTPγS the addition of GTPγS (10 μM).

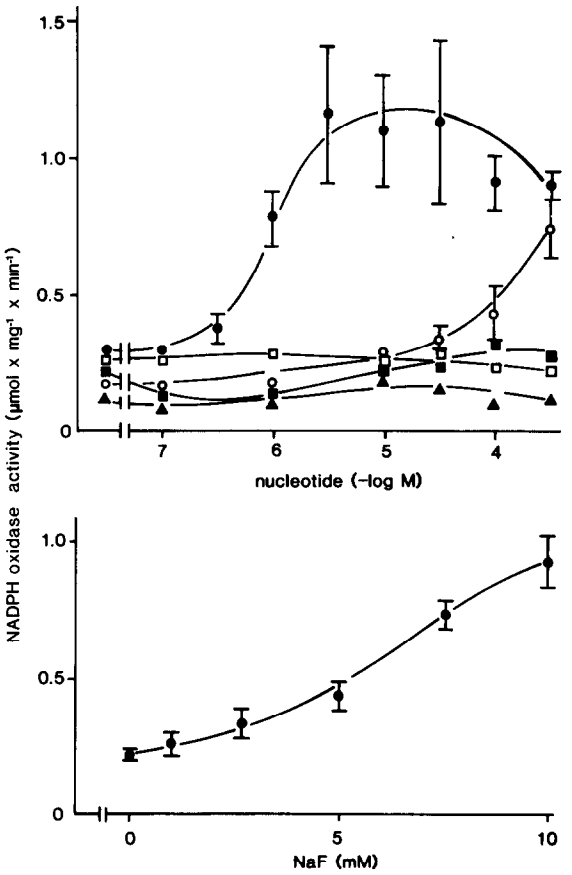

Fig.2. Influence of guanine and adenine nucleotides and of fluoride on NADPH oxidase activity. Upper panel: nucleotides were added to the assay mixture, containing 2 μg membrane protein and 30 μg cytosolic protein, 2 min prior to arachidonic acid (16 μM). Data represent the means ± SE of 3 determinations. (●—●) GTPγS, (○—○) GppNHp, (▲—▲) GTP, (■—■) ATPγS, (□—□) AppNHp. Lower panel: NaF at the indicated concentrations and 5 μM $Al_2(SO_4)_3$ were added prior to arachidonic acid (16 μM). The assays contained 2 μg membrane protein and 30 μg cytosol protein. Data are the means ± SE of 6 determinations.

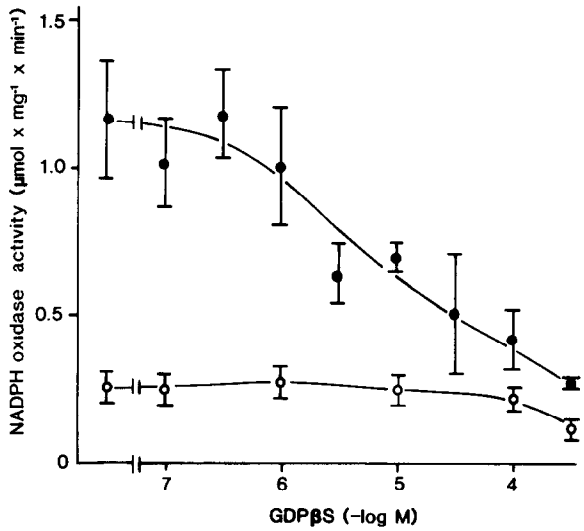

Fig.3. Influence of GDPβS on NADPH oxidase activity in neutrophil membranes. GDPβS was added at the indicated concentrations without (○—○) and with concomitant addition of 1 μM GTPγS (●—●) 2 min prior to arachidonic acid (16 μM). The assays contained 2 μg membrane protein and 30 μg cytosolic protein. Data are the means ± SE of 3 determinations.

non-hydrolyzable GTP analogues (see (fig.2).

In analogy to the adenylate cyclase system [18], the stimulatory effect of GTPγS was not reduced in membranes obtained from neutrophils pretreated with pertussis toxin (fig.4). In contrast to the adenylate cyclase system, in which the effects of GTPγS are significantly delayed following pertussis toxin treatment, the lag time required for NADPH oxidase activation by arachidonic acid and GTPγS in membranes from pertussis toxin-treated neutrophils was not changed.

4. DISCUSSION

It was recently shown that NADPH oxidase in neutrophil membranes is stimulated by arachidonic acid in the presence of neutrophil cytosol [10]. We report here that this stimulation of the enzyme is increased several-fold by stable GTP analogues and fluoride, known stimulators of N-proteins. The present findings suggest that NADPH oxidase represents a new N-protein-regulated effector system. The oxidase represents the first N-protein-controlled enzyme that can be monitored by a photometric method.

In contrast to the current opinion that chemotactic peptides indirectly stimulate NADPH oxidase in the course of neutrophil activation [1], our results suggest a more direct involvement of an N-protein in the regulation of NADPH oxidase. The identity of the cytosolic cofactor required for NADPH oxidase activation in neutrophil membranes and the mechanism by which arachidonic

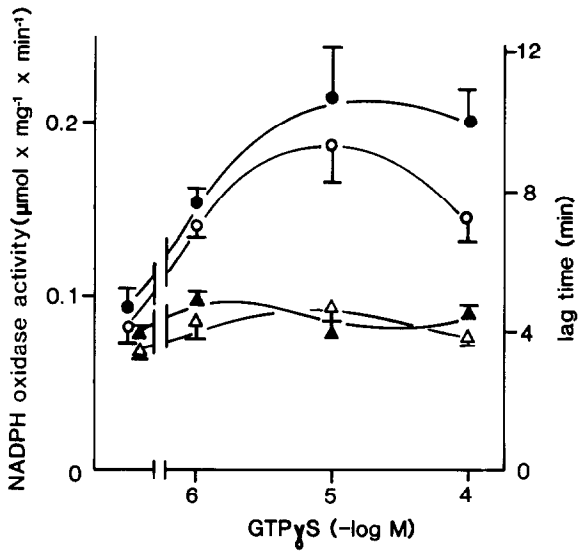

Fig.4. Effect of GTPγS on NADPH oxidase activity in membranes obtained from pertussis toxin-treated neutrophils. GTPγS was added to membranes obtained from neutrophils treated with pertussis toxin or its carrier. The assay mixture contained 7 μg membrane protein and 170 μg cytosolic protein; the reaction was started with arachidonic acid (16 μM). Data are means ± SE of 4 determinations. NADPH oxidase activity is indicated by circles, the lag time from addition of arachidonic acid to reaching maximal velocity (determined as described in fig.1) by triangles. Closed symbols represent pertussis toxin-treated neutrophil membranes, open symbols the controls.

acid stimulates superoxide generation are unknown. Since arachidonic acid stimulates cytosolic protein kinase C [19,20], it has been supposed that protein kinase C may represent the cytosolic cofactor [10]. According to a recent report, an N-protein may be involved in the activation of membrane-associated protein kinase C, because GTPγS (10 μM) stimulates protein kinase C in membranes of rabbit peritoneal neutrophils [21]. On the other hand, arachidonic acid may interact directly with the neutrophil membrane, activating NADPH oxidase by facilitating the electron transport from NADPH to cytochrome b_{-245} or the interaction of the cytosolic component with the enzyme [22]. Therefore, at present it is not yet possible to explain the mechanism by which guanine nucleotides, in concert with arachidonic acid and neutrophil cytosol, stimulate superoxide production in human neutrophil membranes.

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