Guanine nucleotides induce tyrosine phosphorylation and activation of the respiratory burst in neutrophils

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Activation of the NADPH oxidase was examined in electrically permeabilized human neutrophils exposed to non-hydrolysable guanine nucleotides. Guanosine 5'-[γ -thio]triphosphate (GTP[S]) induced a marked increase in the rate of O₂ consumption, which was partially resistant to staurosporine, an inhibitor of protein kinase C, under conditions where the response to diacylglycerol was virtually abolished. The respiratory burst elicited by GTP[S] was dependent on the presence of ATP and Mg²⁺, suggesting involvement of phosphorylation reactions. Accordingly, phosphoprotein formation was greatly stimulated by the guanine nucleotide. The polypeptide phosphorylation pattern induced by GTP[S] was similar to, but not identical with, that observed with diacylglycerol, indicating the activation of kinases other than protein kinase C by the guanine nucleotide. The possible involvement of tyrosine kinases was assessed by immunoblotting using anti-phosphotyrosine antibodies. Treatment of electroporated cells with GTP[S] stimulated the accumulation of tyrosine-phosphorylated proteins. This effect was not induced by diacylglycerol, indicating that tyrosine phosphorylation is not secondary to stimulation of protein kinase C. The results indicate that, in neutrophils, activated G-proteins can stimulate tyrosine kinase and/or inhibit tyrosine phosphatase activity. Changes in the amounts of tyrosine-phosphorylated proteins may signal activation of the respiratory burst.

INTRODUCTION

Neutrophils undergo a respiratory burst when stimulated by a variety of soluble or particulate activators [1,2]. The O₂ consumed is used primarily by a membrane-bound NADPH oxidase to generate the bactericidal superoxide anion. It is generally believed that activation of the respiratory burst through cell-surface receptors is triggered by the stimulation of protein kinase C and/or by the increase in cytosolic Ca²⁺ that follow the hydrolysis of phosphatidylinositol 4,5-bisphosphate [3,4]. However, recent evidence has shown that, for some stimuli, the oxidative burst persists under conditions where protein kinase C is inhibited [5–7] and cytosolic Ca²⁺ is invariant [7], suggesting the existence of additional activation pathways.

With most activators, pretreatment of the cells with pertussis toxin prevents the stimulation of O₂ metabolism, suggesting that activation is mediated by a guanine-nucleotide-binding or G-protein [8,9]. This possibility has been tested further by exposing cell-free systems to non-hydrolysable guanine nucleotides. However, the nucleotides could only enhance activation by detergents or arachidonic acid, but did not by themselves stimulate the NADPH oxidase [10,11]. This failure of the guanine nucleotides to elicit the response is likely to be related to the fact that the cell-free systems used are similarly unable to mount receptor-mediated responses. In the present work, we assessed the role of G-proteins in the activation of the NADPH oxidase, using electrically permeabilized (electroporated) human neutrophils. The

plasma membrane of these cells is rendered permeant to small molecules such as GTP[S]. However, unlike detergent-permeabilized cells and the available cell-free systems, electroporated cells retain the ability to be stimulated through cell-surface receptors [7], indicating that the processes that follow receptor occupancy remain functional. The results demonstrate that exposure of permeabilized cells to GTP[S] in the absence of other exogenous stimuli activated the respiratory burst. Activation was found to be ATP- and Mg²⁺-dependent and accompanied by an increase in tyrosine phosphorylation. Therefore tyrosine phosphorylation may represent an alternative pathway for activation of the NADPH oxidase.

EXPERIMENTAL

Materials

GTP (Li⁺ salt), p[NH]ppG (Na⁺ salt), ATP (K⁺ salt), p[NH]ppA (Li⁺ salt), NADPH (Na⁺ salt) and RPMI 1640 medium were from Sigma. Staurosporine was from Seikagaku America Inc. (St. Petersburg, FL, U.S.A.). [γ -³²P]ATP was from ICN Radiochemicals, and bovine serum albumin from Calbiochem.

Solutions

RPMI 1640 (bicarbonate-free, 25 mM-Hepes) was buffered at pH 7.3. Permeabilization medium contained (mM) 140 KCl, 10 Hepes, 1 EGTA and 0.193 CaCl₂ (to give a final free [Ca²⁺] of 100 nM), and, unless otherwise

Abbreviations used: p[NH]ppA, p[NH]ppG, adenosine (or guanosine) 5'-[β -imido]triphosphate; DiC₈, 1,2-dioctanoyl-*sn*-glycerol; CDTA, *trans*-1,2-diaminocyclohexane-*NNN'*-tetra-acetic acid; ATP[S], GTP[S], adenosine (or guanosine) 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; PAGE, polyacrylamide-gel electrophoresis.

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indicated, 10 glucose, 1 MgCl_2 and 1 ATP. Medium was adjusted to pH 7.0. All media were adjusted to 290 ± 5 mosm.

Cell isolation and permeabilization

Neutrophils were isolated from fresh heparinized human blood by dextran sedimentation followed by Ficoll-Hypaque gradient centrifugation [12]. Contaminating red cells were removed by NH_4Cl lysis. Cells were counted with a Coulter Counter model ZM, and resuspended in Hepes-buffered RPMI 1640 at 10^7 cells/ml. The cells were maintained in this medium at room temperature until use.

Permeabilization was performed as outlined in [7]. Briefly, 8×10^6 cells were washed and resuspended in 0.8 ml of ice-cold permeabilization medium. Cells were permeabilized with two discharges of 5 kV/cm from a 25 μF capacitor by using a Bio-Rad Gene Pulser. The cells were washed in ice-cold permeabilization medium between discharges. Permeabilized cells were either used immediately or maintained on ice for up to 15 min.

O_2 consumption

O_2 consumption was measured polarographically with a model 53 biological oxygen monitor (Yellow Springs Instrument Co.); 4×10^6 cells were permeabilized and resuspended in 2 ml of permeabilization medium containing 2 mM-NADPH at 37°C with stirring. O_2 consumption was calculated by using a solubility coefficient of 0.024 ml of O_2 /ml at 37°C .

Phosphoprotein formation

Some 4×10^6 cells were permeabilized and suspended in 1 ml of permeabilization medium containing 50 μM -ATP and 2 mM-NADPH at 37°C . When indicated, 10 nM-staurosporine was added at this stage. After 2 min, 25 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was added, and, 10 s later, the cells were stimulated with GTP[S] or DiC_8 , as indicated. After 2 min, 100 μM -phenylmethanesulphonyl fluoride was added and the cells were sedimented, resuspended in SDS/mercaptoethanol sample buffer, and boiled. SDS/PAGE was carried out on 10%-acrylamide gels [13]. Gels were silver-stained, dried, and autoradiograms exposed for 4–5 days with intensifying screens at -20°C .

Anti-phosphotyrosine antibodies and immunoblots

Samples were prepared as for phosphoprotein formation, except that the medium contained 1 mM-ATP and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was omitted. Rabbit affinity-purified polyclonal phosphotyrosine-specific antibodies or monoclonal phosphotyrosine-specific antibodies were kindly given by Dr. M. P. Kamps and Dr. B. M. Sefton. Similar results were obtained with a polyclonal antibody prepared by G.B.M. by the method of Kamps & Sefton [14]. Immunoblotting was performed as described [14]. Briefly, sites that had not reacted were blocked with 5% bovine serum albumin and 1% ovalbumin. The blot was incubated with phosphotyrosine-specific antibodies and washed. The monoclonal anti-phosphotyrosine antibody was followed by goat anti-mouse antibody. The nitrocellulose filters were incubated with 1 μCi of ^{125}I -labelled Protein A, washed and used for autoradiography.

Statistics

All experiments were performed at least three times with blood from different donors. Results are presented

as representative traces or autoradiograms or as means ± 1 S.E.M.

RESULTS AND DISCUSSION

The activity of the NADPH oxidase in human neutrophils was measured as the rate of O_2 consumption. When 10 μM -GTP[S] was added to a suspension of electrically permeabilized cells, the rate of O_2 consumption increased markedly after a lag of approx. 0.5 min (Fig. 1), attaining a maximum of 10.3 ± 0.6 nmol of O_2 /min per 10^6 cells ($n = 26$). Unlike observations in cell-free systems [10,11], stimulation of the permeabilized cells by guanine nucleotides did not require the presence of other activators or detergents. The stimulation was strictly dependent on the presence of NADPH and persisted for at least 5 min, the rate decreasing only slowly over this period. O_2 consumption was also activated by 50 μM -p[NH]ppG and by a combination of 5 mM-NaF and 25 μM - AlCl_3 , reaching maximal rates of 10.2 ± 1.8 ($n = 4$) and 5.9 ± 0.5 ($n = 5$) nmol of O_2 /min per 10^6 cells respectively. The addition of 100 μM -GDP[S] inhibited the response elicited by

Fig. 1. ATP- and Mg^{2+} -dependence of GTP[S]-stimulated O_2 consumption in permeabilized neutrophils

Cells were suspended in permeabilization medium containing 2 mM-NADPH 1–2 min before the start of the trace, and O_2 consumption was measured with an oxygen electrode as outlined in the Experimental section: 10 μM -GTP[S], 200 μM -ATP or 1 mM- MgCl_2 was added where indicated. Initially, the media contained: (a) 1 mM-ATP, 10 mM-glucose, 1 mM- MgCl_2 ; (b) 0 ATP, 0 glucose, 1 mM- MgCl_2 ; (c) 0 ATP, 0 glucose, 0 Mg^{2+} and 10 μM -CDTA. Traces shown are representative of at least three experiments.

1 μM -GTP[S] by $97 \pm 3.3\%$ ($n = 3$). Under comparable conditions, 100 μM -ADP inhibited only 10% of the response. In intact neutrophils, O_2 consumption was not affected by up to 100 μM -GTP[S], indicating that the site of action of the nucleotide is intracellular. Activation of the oxidase by non-hydrolysable GTP analogues acting intracellularly and by AlF_4^- , and inhibition of this effect by GDP[S], are indicative of mediation by a G-protein.

When cells were permeabilized and stimulated with GTP[S] in the absence of exogenously added ATP, a markedly decreased response was observed after a lag of over 1 min (Fig. 1b). This small stimulation may reflect the presence of residual intracellular ATP, or the existence of an ATP-independent component of the response. Addition of 200 μM -ATP to the medium restored the response (Fig. 1b). The non-cleavable ATP analogue p[NH]ppA did not support activation, indicating that ATP hydrolysis is required. As shown in Fig. 1(c), GTP[S] failed to induce a respiratory burst in a medium lacking both ATP and Mg^{2+} and containing the Mg^{2+} chelator CDTA (10 μM). Addition of 200 μM -ATP alone was not sufficient to restore the response, but the subsequent addition of Mg^{2+} was followed by stimulation of O_2 consumption.

The finding that ATP plus Mg^{2+} are required for the full expression of the GTP[S]-induced respiratory burst suggests the involvement of phosphotransferase reactions. We therefore investigated the possible occurrence of protein phosphorylation upon treatment of the cells with the guanine nucleotide. Electroporated cells were incubated in the presence of [$\gamma\text{-}^{32}\text{P}$]ATP and treated with or without GTP[S]. The reaction was then stopped and the phosphoprotein composition of the cell extract was analysed by gel electrophoresis, followed by autoradiography. As illustrated in Fig. 2, a marked enhancement in protein phosphorylation was apparent in cells treated with GTP[S]. Multiple polypeptides, with molecular masses ranging from 30 to 200 kDa, displayed enhanced phosphorylation. These observations suggest that phosphoprotein formation is involved in the GTP[S]-stimulated burst of O_2 consumption, accounting for its ATP- and Mg^{2+} -dependence.

It is well established that, in neutrophils, G-proteins mediate the activation of phospholipase C upon engagement of surface receptors by activators [15]. The resultant hydrolysis of phosphoinositides yields diacylglycerols that can activate protein kinase C [3,16]. In intact cells, stimulation of this kinase by phorbol esters suffices to elicit the respiratory burst [4]. It was therefore important to determine whether this pathway could account for the effects of GTP[S] in permeabilized cells. As reported earlier [7], addition of 1,2-dioctanoyl-*sn*-glycerol (DiC_8), an exogenous diacylglycerol, induced a marked burst of O_2 consumption in electroporated cells (Fig. 3a). Concomitantly, DiC_8 stimulated protein phosphorylation (Fig. 2). Indeed, many of the polypeptides phosphorylated in response to GTP[S] were also phosphorylated by addition of DiC_8 . However, two findings indicate that the functional effects of the guanine nucleotide are not entirely attributable to formation of diacylglycerol. First, the phosphorylation patterns of samples treated with GTP[S] and DiC_8 , though similar, are not identical. For instance, polypeptides of molecular mass 47 kDa and 55 kDa are labelled much more intensely in DiC_8 -treated cells. Conversely, a polypeptide of molecular mass 58 kDa is more apparent after treatment with GTP[S].

Fig. 2. Protein phosphorylation stimulated by GTP[S] or DiC_8 in permeabilized neutrophils

Permeabilized cells were activated in the presence of [$\gamma\text{-}^{32}\text{P}$]ATP as outlined in the Experimental section: 10 μM -GTP[S], 1.5 μM - DiC_8 or 10 nM-staurosporine was added where indicated. Left, silver staining; right, autoradiogram. Numbers indicate the molecular mass, in kDa. All the lanes presented are from a single gel, but were cut and re-arranged to omit extraneous lanes. Results shown are representative of three experiments.

Fig. 3. Effect of staurosporine on O_2 consumption in permeabilized neutrophils

(a) 10 μM -GTP[S] or 1.5 μM - DiC_8 added at the arrow; (b) 10 nM-staurosporine added at the first arrow and either GTP[S] or DiC_8 at the second arrow. All media contained 1 mM-ATP. Other conditions were as for Fig. 1.

Secondly, the respiratory burst elicited by the two stimuli was differentially susceptible to inhibition by staurosporine, a potent, albeit not entirely specific, inhibitor of protein kinase C [17]. As shown in Fig. 3, staurosporine

blocked the response to DiC_8 almost entirely ($93 \pm 0.7\%$ inhibition; $n = 3$). In contrast, the response to GTP[S] , though substantially decreased ($73 \pm 5.5\%$ inhibition; $n = 6$), was more resistant to the blocker. Thus, whereas in the absence of inhibitor the response to DiC_8 was approximately double that of GTP[S] (maximal rates of 23 ± 3 and 10.3 ± 0.6 nmol of O_2/min per 10^6 cells respectively), the latter was larger in the presence of staurosporine. Similar results were obtained with 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H7), a different protein kinase C inhibitor (results not shown).

Consistent with the inhibition of the respiratory burst, the presence of staurosporine during stimulation markedly decreased the phosphorylation induced by both GTP[S] and DiC_8 (Fig. 2). Though in some experiments the phosphorylation of certain polypeptides by GTP[S] appeared to be less sensitive to staurosporine than in cells stimulated by DiC_8 , this was not a consistent observation. More often, the patterns observed with both stimuli in the presence of staurosporine (or H7) were virtually indistinguishable (e.g. Fig. 2). Two possible explanations could account for the differential susceptibility of the respiratory burst to staurosporine, despite the similarity of the phosphorylation patterns. Protein phosphorylation may be unrelated to the staurosporine-resistant component of the respiratory burst induced by the guanine nucleotide. Alternatively, phosphorylation of a minor component, undetectable by total phosphorylation studies, may be responsible for the staurosporine-resistant activation of the NADPH oxidase.

Tyrosine phosphorylation plays a central role in signal transduction by several polypeptide-hormone receptors [18]. In general, the tyrosine-phosphorylated protein(s) responsible for coupling the receptor with the appropriate effectors represent only a minor fraction of the total phosphoprotein content of the cells. Consequently, in whole cell extracts, they are difficult to detect unless the different phosphoamino acids are separated or otherwise identified. It was therefore conceivable that the stimulation of the respiratory burst by GTP[S] was mediated by formation of small amounts of tyrosine-phosphorylated proteins, not detectable by experiments like those in Fig. 2. This possibility was studied by analysing protein-tyrosine phosphorylation by immunoblotting, by using antibodies specific for phosphotyrosine. The results of two such experiments are shown in Fig. 4. Otherwise untreated permeabilized neutrophils displayed comparatively small amounts of tyrosine-phosphorylated polypeptides of molecular masses 44, 57 and 80 kDa. Treatment of the permeabilized cells with GTP[S] induced a marked enhancement of protein-tyrosine phosphorylation. Major bands of molecular masses 44, 57, 72, 80, 90–110 and 180 kDa were found to be tyrosine-phosphorylated upon treatment with the nucleotide. Similar results were obtained by using polyclonal (Fig. 4a) and monoclonal (Fig. 4b) anti-phosphotyrosine antibodies, validating the identification of tyrosine-phosphorylated proteins. The specificity of the labelling was further indicated by the finding that, except for a band of molecular mass 23 kDa, an excess of free phosphotyrosine (2 mM) virtually eliminated binding of the antibody. In contrast, labelling was essentially unaffected by equivalent concentrations of phosphoserine and phosphothreonine (results not shown).

The tyrosine phosphorylation induced by GTP[S] was not a consequence of the stimulation of protein kinase C

Fig. 4. Anti-phosphotyrosine immunoblot of permeabilized cells stimulated with GTP[S] or DiC_8 .

Samples were prepared as outlined in the Experimental section. (a) Samples were analysed by electrophoresis on a SDS/7%-polyacrylamide gel and immunoblotted by using a polyclonal anti-phosphotyrosine antibody; (b) samples were analysed by electrophoresis on a 10%-acrylamide gel and immunoblotted by using a monoclonal anti-phosphotyrosine antibody. Numbers indicate the molecular mass, in kDa. Where indicated, the cells were stimulated with $10 \mu\text{M}$ - GTP[S] or $1.5 \mu\text{M}$ - DiC_8 .

by endogenous diacylglycerol. This was evidenced by the failure of DiC_8 to increase the extent of tyrosine phosphorylation (Fig. 4a) under conditions where O_2 consumption and ^{32}P incorporation were greatly stimulated. This is consistent with the recent finding that phorbol esters had no detectable effect on tyrosine phosphorylation in intact cells [19]. Thus, in neutrophils, G-proteins are capable of inducing protein phosphorylation at tyrosine residues by a mechanism independent of protein kinase C. It is noteworthy that the tyrosine phosphorylation induced by GTP[S] was somewhat inhibited by staurosporine (results not shown). In view of the results above, this probably represents a non-specific effect of staurosporine which is unrelated to inhibition of protein kinase C, the putative target of the blocker.

Nucleoside-diphosphate kinase activity has been reported in HL-60 leukaemic cells and could catalyse the formation of ATP[S] from GTP[S] and ADP [20]. ATP[S] may then have served as the substrate of protein-tyrosine kinase. The increase in tyrosine phosphorylation seen upon exposure to GTP[S] could therefore result from the formation of thiophosphoproteins, which are known to be resistant to hydrolysis by phosphatases. However, permeabilized cells stimulated with GTP[S] in the absence of ATP and the presence of ATP[S] failed to show any increase in tyrosine phosphorylation when analysed in an immunoblot by using the polyclonal anti-phosphotyrosine antibody, suggesting that the antibody does not recognize thiophosphoproteins. These observations also rule out the possibility that GTP[S] itself was used as a substrate for tyrosine kinases.

In summary, addition of GTP[S] to permeabilized cells was found to stimulate O_2 consumption, protein phosphorylation in general and tyrosine phosphorylation in particular. These effects are most likely due to activation of a G-protein, inasmuch as they: (a) require entry of the nucleotide into the cell; (b) are specific to non-hydrolysable guanine nucleotides; and (c) are antagonized by GDP[S]. The activation of G-proteins by non-hydrolysable guanine nucleotides is likely to resemble the physiological activation by ligand-receptor interaction. This is suggested by the pertussis-toxin sensitivity of the responses elicited by a variety of stimuli (see the Introduction). Moreover, binding of chemotactic peptide to its receptor stimulates not only O_2 consumption and phosphoprotein formation, but also the accumulation of tyrosine-phosphorylated polypeptides of molecular masses 62 kDa and 125 kDa [19]. Polypeptides of comparable size were tyrosine-phosphorylated in permeabilized cells treated with GTP[S].

The nature of the kinase(s) responsible for G-protein-induced tyrosine phosphorylation is not known. A haemopoietic-cell protein-tyrosine kinase (*hck*) has been recently detected in granulocytes, where it is particularly abundant [21,22]. This kinase is related to *c-src* and is therefore likely to be a peripheral membrane protein. The mode of activation of *hck* is not understood, but it is conceivable that G-proteins can directly or indirectly stimulate it. Alternatively, the kinase may be constitutively active, and enhanced phosphorylation may result from inhibition of tyrosine phosphatases. In this regard, it is noteworthy that the presence of vanadate, a potent tyrosine phosphatase inhibitor, produced the accumulation of tyrosine-phosphorylated proteins even in unstimulated permeabilized cells (P. E. Nasmith, W. Furuya, G. B. Mills & S. Grinstein, unpublished work). Moreover, a small yet significant inhibition of tyrosine phosphatase activity was detected in stimulated neutrophils [23].

It is not clear whether protein-tyrosine phosphorylation mediates the activation of the NADPH oxidase in cells treated with GTP[S]. Both the respiratory burst and accumulation of phosphotyrosine are evident within minutes of addition of the guanine nucleotide, require ATP and Mg^{2+} , and are only partially sensitive to staurosporine at the concentrations used, consistent with a causal relationship. In addition, the observation that vanadate induces a large O_2 -consumption burst in permeabilized cells (D. J. Lu, W. Furuya & S. Grinstein, unpublished work) is also consistent with a role of tyrosine-phosphorylated proteins in signal transduction. Despite this suggestive evidence, confirmation of a role for tyrosine phosphorylation in neutrophil activation must await the development of specific tyrosine kinase inhibitors, kinase-deficient mutants or the reconstitution of receptor-mediated responses in cell-free systems where

the kinase can be manipulated. It also remains to be elucidated whether activation of tyrosine phosphorylation by G-proteins is exclusive to neutrophils or is instead a general signal-transduction mechanism.

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