The role of nucleoside-diphosphate kinase reactions in G protein activation of NADPH oxidase by guanine and adenine nucleotides

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NADPH-oxidase-catalyzed superoxide (O_2^-) formation in membranes of HL-60 leukemic cells was activated by arachidonic acid in the presence of Mg^{2+} and HL-60 cytosol. The GTP analogues, guanosine 5'-[γ-thio]triphosphate (GTP[γS] and guanosine 5'-[β,γ-imido]triphosphate, being potent activators of guanine-nucleotide-binding proteins (G proteins), stimulated O_2^- formation up to 3.5-fold. The adenine analogue of GTP[γS], adenosine 5'-[γ-thio]triphosphate (ATP[γS]), which can serve as donor of thiophosphoryl groups in kinase-mediated reactions, stimulated O_2^- formation up to 2.5-fold, whereas the non-phosphorylating adenosine 5'-[β,γ-imido]triphosphate was inactive. The effect of ATP[γS] was half-maximal at a concentration of 2 µM, was observed in the absence of added GDP and occurred with a lag period two times longer than the one with GTP[γS]. HL-60 membranes exhibited nucleoside-diphosphate kinase activity, catalyzing the thiophosphorylation of GDP to GTP[γS] by ATP[γS]. GTP[γS] formation was half-maximal at a concentration of 3 – 4 µM ATP[γS] and was suppressed by removal of GDP by creatine kinase/creatine phosphate (CK/CP). The stimulatory effect of ATP[γS] on O_2^- formation was abolished by the nucleoside-diphosphate kinase inhibitor UDP. Mg^{2+} chelation with EDTA and removal of endogenous GDP by CK/CP abolished NADPH oxidase activation by ATP[γS] and considerably diminished stimulation by GTP[γS]. GTP[γS] also served as a thiophosphoryl group donor to GDP, with an even higher efficiency than ATP[γS]. Transthiophosphorylation of GDP to GTP[γS] by GTP[γS] was only partially inhibited by CK/CP. Our results suggest that NADPH oxidase is regulated by a G protein, which may be activated either by exchange of bound GDP by guanosine triphosphate or by thiophosphoryl group transfer to endogenous GDP by nucleoside-diphosphate kinase.

Human neutrophils and HL-60 leukemic cells possess a plasma-membrane-bound NADPH oxidase, which catalyzes O_2^- formation and can be activated by chemotactic peptides [1 – 3]. In addition, chemotactic peptides activate phospholipase-C-catalyzed release of inositol triphosphate and diacylglycerol from polyphosphoinositol lipids, thus leading to calcium mobilization and to the activation of protein kinase C. Furthermore, these peptides activate phospholipase-A_2-mediated release of arachidonic acid [4, 5]. As arachidonic acid induces O_2^- formation in intact cells [2, 6] and in cell-free systems [2, 7 – 12], it was suggested that arachidonic acid may serve as an intracellular messenger for NADPH oxidase activation. Stimulation of the enzyme in cell-free systems de-

pends on Mg^{2+} and on a cytosolic cofactor, which appears to be different from protein kinase C [2, 9, 12]. However, some phosphorylation reactions may play a role in NADPH oxidase activation, as ATP is required for maximal rates of O_2^- generation [9, 11]. We and others have recently reported that NADPH oxidase activity was enhanced severalfold by GTP analogues, but not by the corresponding ATP anaolgues, suggesting that a guanine-nucleotide-binding (G protein) is involved in the activation process of NADPH oxidase [2, 8 – 10, 13].

There is evidence that nucleoside-diphosphate kinase (NDPK), catalyzing phosphate group transfer from NTP to NDP [14], is involved in the activation of G proteins, i.e. G_s and G_i, the G proteins mediating hormonal stimulation and inhibition of adenylyl cyclase respectively [13, 15 – 18], and G_p, the G protein assumed to activate phospholipase C [19]. It has recently been shown that NDPK is closely associated with certain G proteins in HeLa S3 and Ehrlich ascites tumor cells [20, 21]. In addition, NDPK has been reported to catalyze the transfer of phosphate groups to GDP bound to various G proteins [16, 20, 21]. These findings prompted us to study the role of NDPK reactions in the activation of NADPH oxidase by G proteins. We report here that HL-60 membranes possess NDPK activity, catalyzing the formation of GTP[γS] from ATP[γS] and, thereby, promoting activation of G proteins and of NADPH oxidase.

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MATERIALS AND METHODS

Materials

Creatine kinase (CK), creatine phosphate (CP), super-oxide dismutase, ferricytochrome c (Type III) and arachidonic acid (grade I, 99% pure) were obtained from Sigma Chemie (Taufkirchen, FRG). A stock solution of arachidonic acid (16.4 mM) in 100% ethanol was prepared under nitrogen and stored at $-20\,°C$. All unlabelled nucleotides were obtained from Boehringer Mannheim (Mannheim, FRG). [8-^{3}H]GDP (0.43 TBq/mmol) was from Amersham Buchler (Braunschweig, FRG). Poly(ethyleneimine)-cellulose F thin-layer chromatography sheets (layer thickness 0.1 mm) were from E. Merck (Darmstadt, FRG).

Cell culture and cell fractionation

Cell culture media were obtained from Biochrom (Berlin, FRG). HL-60 cells were grown in suspension culture in RPMI-1640 medium supplemented with 10% (by vol.) horse serum, 1% (by vol.) non-essential amino acids, 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin at 37 °C in a humidified atmosphere with 7% CO_2. To induce myeloid differentiation, HL-60 cells were seeded at a density of 10^6 cells/ml and were cultured for 5 days in the medium supplemented with 1.25% (by vol.) dimethylsulfoxide [3]. HL-60 membranes and cytosol were prepared as described recently [2, 22].

Assay for NADPH oxidase activity

NADPH-oxidase-catalyzed O_2^- formation was monitored by continuous measurement of ferricytochrome c reduction inhibitable by superoxide dismutase using an Uvikon 810 dual-beam spectrophotometer (Kontron, Eching, FRG) [2, 9]. Reaction mixtures (500 µl) contained 55 µg membrane protein, 78 µg cytosolic protein, 500 µM NADPH, 10 µM FAD, 100 µM ferricytochrome c, $MgCl_2$ (2 mM free Mg^{2+}), 20 mM KH_2PO_4, 40 mM KCl and 20 mM triethanolamine/HCl, pH 7.0. Reference cuvettes contained all the components listed above plus 50 µg superoxide dismutase. Assay mixtures were preincubated for 2 min at 25 °C in the absence or presence of facultative additions. Reactions were initiated by the addition of arachidonic acid (200 µM). V_{max} of O_2^- formation was calculated from the linear parts of absorbance curves. The presence of KCN did not affect O_2^- generation.

Assay for NDPK activity

The assay mixtures for measurement of GTP[γS] formation (50 µl) contained 0.5 µM [8-^{3}H]GDP (37 kBq/tube), unlabelled ATP[γS] or GTP[γS] at the indicated concentrations, 2 mM $MgCl_2$, 50 mM triethanolamine/HCl, pH 7.6 and facultative additions. Reactions were initiated by the addition of HL-60 membranes (40 µg protein/tube) and conducted for 10 min or the indicated periods of time at 25 °C. Reactions were terminated by the addition of EDTA to give a final concentration of 17 mM in a volume of 60 µl. The assay tubes were then centrifuged for 3 min at 8800 × g. 10 µl resultant supernatant fluid together with 0.5 µl solution containing unlabelled GTP[γS], GTP and GDP (final concentration 1 mM each) were spotted onto poly(ethyleneimine)-cellulose thin-layer chromatography sheets, containing a fluorescence indicator. The nucleotides were separated by developing the plates for 14 cm at room temperature in 0.75 M
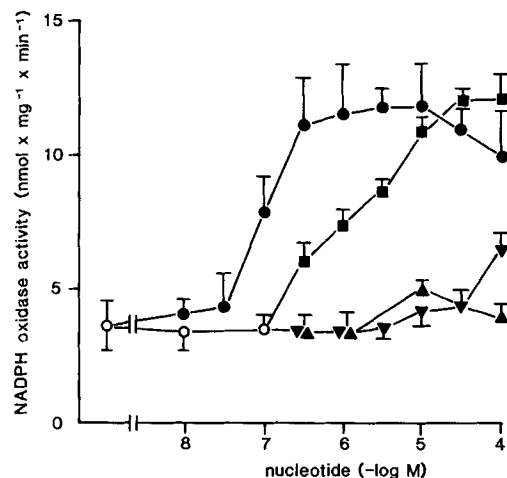

Fig. 1. *Influence of various guanine nucleotides on NADPH oxidase activity in HL-60 membranes.* NADPH oxidase activity was determined in membranes of HL-60 cells as described in Materials and Methods in the presence of various guanine nucleotides at the concentrations indicated. Nucleotides were added to reaction mixtures 2 min prior to arachidonic acid (200 µM). Data are the mean ± SEM of three to twelve experiments. (▲) GTP, (●) GTP[γS], (■) [β,γ-NH]GTP, (▼) [β,γ-CH$_2$]GTP

KH_2PO_4. Nucleotides were visualized by ultraviolet light absorption at 254 nm, and the areas containing GTP[γS] (R_f = 0.05), GTP (R_f = 0.20) and GDP (R_f = 0.42) were scraped off. Nucleotides were eluted from the poly(ethyleneimine)-cellulose by vigorous shaking of the samples for 1 h in 750 µl buffer containing 0.7 M $MgCl_2$ and 20 mM Tris/HCl, pH 7.5. After centrifugation for 3 min at 1000 × g, 500 µl supernatant fluid were added to a Triton X-100/Triton X-114/xylene-based scintillation fluid, and the radioactivity was determined in a liquid scintillation spectrometer.

RESULTS

O_2^- formation in membranes of HL-60 cells was activated by arachidonic acid in the presence of Mg^{2+} and HL-60 cytosol; this basal O_2^- was stimulated by guanine nucleotides (Fig. 1): While GTP itself was rather inactive, its anlogues, GTP[γS] and [β,γ-NH]GTP [13, 23], enhanced O_2^- generation up to 3.5-fold with half-maximal effects occurring at concentrations of 0.1 µM and 1 µM respectively. In contrast, [β,γ-CH$_2$]GTP was far less effective and enhanced O_2^- generation only at concentrations above 30 µM. This order of potency of guanine nucleotides to stimulate NADPH oxidase is in agreement with that obtained for activation of various G proteins [13], supporting the view that NADPH oxidase is regulated by a G protein.

The adenine analogue of GTP[γS], ATP[γS], which can serve as a thiophosphoryl group donor in kinase-mediated reactions [23], also enhanced O_2^- formation (Fig. 2). Maximal activation by ATP[γS] (2.5-fold) was observed at a concentration of 10 µM, with a half-maximal stimulation occurring at a concentration of 2 µM. However, even when added at a supramaximally stimulatory concentration (100 µM), stimulation by ATP[γS] did not reach the maximal enhancement of enzyme activity obtained with GTP[γS]. In contrast to ATP[γS], ATP, ATP[αS] and [β,γ-NH]ATP employed at concentrations up to 100 µM did not stimulate NADPH oxidase. The difference between ATP[γS] and the other adenosine
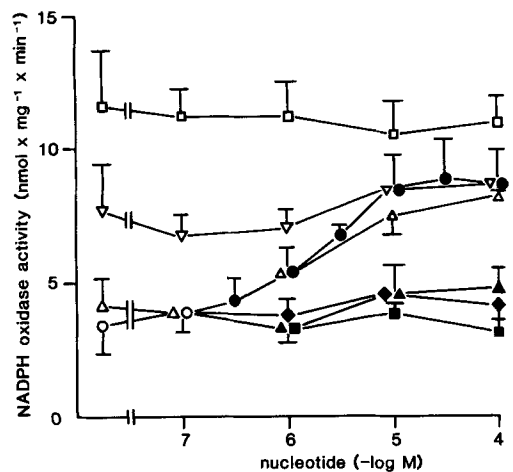
Fig. 2. *Influence of various adenine nucleotides on NADPH oxidase activity in HL-60 membranes: interaction of ATP[γS] with GTP[γS].* NADPH oxidase activity was determined in membranes of HL-60 cells in the presence of various adenine nucleotides at the indicated concentrations. In addition, the effect of ATP[γS] was studied in the presence of three fixed concentrations of GTP[γS]. Nucleotides were added to reaction mixtures 2 min prior to arachidonic acid (200 μM). Data represent the mean ± SEM of three to twelve experiments. (▲) ATP, (●) ATP[γS], (◆) ATP[αS], (■) [β,γ-NH]ATP, (△) ATP[γS] plus GTP[γS] (30 nM), (▽) ATP[γS] plus GTP[γS] (100 nM), (□) ATP[γS] plus GTP[γS] (1 μM)

triphosphates is that ATP and ATP[αS] may deliver phosphoryl groups and [β,γ-NH]ATP is inactive in this regard [23, 24]. These data suggested that endogenous GDP served as a thiophosphoryl group acceptor in a NDPK reaction, leading to the formation of GTP[γS]. Therefore, the interaction of ATP[γ]S and GTP[γ]S on NADPH oxidase was studied. GTP[γS] at a subthreshold concentration (30 nM) did not affect NADPH oxidase activation by ATP[γS]. When GTP[γS] was present at half-maximally and maximally stimulatory concentrations (0.1 μM and 1 μM respectively), ATP[γS] did not further enhance NADPH oxidase activity, suggesting that ATP[γS] and GTP[γS] stimulate NADPH oxidase by a common mechanism.

NADPH oxidase activation by ATP[γS] and GTP[γS] exhibited differential sensitives to various substances (Table 1). NDPK activity depends on the presence divalent cations such as Mg^{2+} [14]. Mg^{2+} chelation by EDTA reduced basal O_2^- generation and abolished the stimulatory effect of ATP[γS], whereas GTP[γS] still caused a twofold stimulation of NADPH oxidase activity. NDP at concentrations in the millimolar range can act as inhibitor of NDPK by formation of an abortive enzyme-NDP complex [14, 15, 18, 25]. When added at a concentration of 1 mM, UDP and ADP (data with ADP not shown) completely suppressed the stimulatory effect of ATP[γS] on O_2^- formation, whereas stimulation by GTP[γS] was not affected. The inhibitory effects of UDP and ADP were only observed when added to reaction mixtures together with ATP[γS], but not when added several minutes after ATP[γS] (data not shown), i.e. when the formation of sufficient amounts of functionally active GTP[γS] was apparently completed. Addition of GDP (0.1 – 3 μM) did not further enhance the stimulatory effect of ATP[γS]. At concentrations above 3 μM, GDP inhibited O_2^- formation, probably by direct inactivation of G proteins [2]. The addition of the NTP-regenerating system CK/CP, which catalyzes the phosphorylation of NDP to NTP [26], prevented ATP[γS]-induced NADPH oxi-

Table 1. *Differential effects of various substances on basal, ATP[γS]-stimulated and GTP[γS]-stimulated NADPH oxidase activity in HL-60 membranes*

2 min prior to the addition of arachidonic acid (200 μM), various agents were added to reaction mixtures in the absence (control) or presence of either ATP[γS] or GTP[γS]. Data are the mean ± SEM of three to twelve experiments. CK, 50 μg creatine kinase; CP, 5 mM creatine phosphate

Addition	NADPH oxidase activity		
	control	ATP[γS] (100 μM)	GTP[γS] (1 μM)
	nmol mg^{-1} min^{-1}		
2 mM Mg^{2+}	3.6 ± 1.0	8.8 ± 1.3	11.5 ± 2.0
5 mM EDTA, no Mg^{2+}	2.3 ± 1.1	2.1 ± 0.7	4.6 ± 0.6
2 mM Mg^{2+} + 1 mM UDP	2.6 ± 0.6	2.7 ± 0.1	11.9 ± 4.5
2 mM Mg^{2+} + 1 μM GDP	3.6 ± 0.8	8.9 ± 0.5	9.5 ± 1.0
2 mM Mg^{2+} + 1 mM ATP	4.9 ± 1.0	5.0 ± 0.2	11.9 ± 4.0
2 mM Mg^{2+} + CK/CP	2.2 ± 0.2	2.5 ± 0.7	4.9 ± 1.0

Table 2. *Formation of [8-³H]GTP[γS] by HL-60 membranes and its modification by a NTP-regenerating system*

Formation of [8-³H]GTP[γS] by HL-60 membranes was measured as described in Materials and Methods with 0.5 μM [8-³H]GDP and either ATP[γS] or GTP[γS] as substrates in the absence (control) or presence of 20 μg creatine kinase plus 5 mM creatine phosphate. Data are the mean ± SD of three experiments

Addition	GTP[γS] formation from	
	ATP[γS] (50 μM)	GTP[γS] (50 μM)
	pmol mg^{-1} $(10 min)^{-1}$	
None	12.80 ± 1.00	18.30 ± 0.05
CK/CP	1.20 ± 0.25	12.80 ± 0.05

dase activation. CK/CP also considerably reduced, although did not abolish, basal as well as GTP[γS]-stimulated O_2^- generation. ATP at a concentration of 1 mM stimulated O_2^- generation by 36% (see Table 2), whereas [β,γ-NH]ATP reduced O_2^- formation by 15% (data not shown). At an ATP concentration of 1 mM, the stimulatory effect of ATP[γS] but not that of GTP[γS] on O_2^- formation was abolished.

The kinetics of NADPH oxidase activation by GTP[γS] and ATP[γS] were different, too (Fig. 3). As reported before [2], arachidonic acid reversibly activated NADPH oxidase in HL-60 membranes. GTP[γS] (1 μM) reinitiated O_2^- formation with a lag period of 100 ± 30 s and a V_{max} of 1.5 nmol O_2^- mg^{-1} min^{-1}. ATP[γS] (100 μM) also reinitiated O_2^- formation but, compared to GTP[γS], with a two times longer lag period and an about 30% reduced V_{max} value. The lag periods required to reach V_{max} with 1 μM GTP[γS] or with 100 μM ATP[γS], added prior to arachidonic acid, amounted to 120 ± 30 s and 230 ± 80 s (mean ± SEM of 12 experiments) respectively. UDP prevented reinitiation of O_2^- formation by ATP[γS] (see Fig. 3) but not by GTP[γS] (data not shown), and addition of GTP[γS] subsequent to ATP[γS] plus UDP reinitiated O_2^- formation. The addition of GTP[γS] and ATP[γS] subsequent to ATP[γ]S and GTP[γS], respectively, did not reactivate NADPH oxidase.
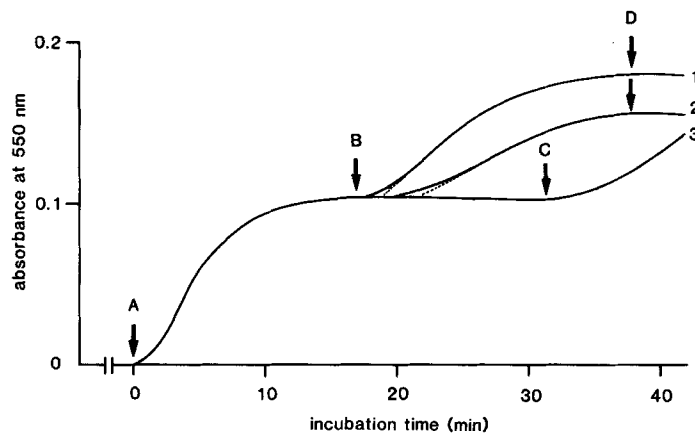
Fig. 3. *Reactivation of arachidonic-acid-induced O_2^- formation by GTP[γS] and ATP[γS].* A, the addition of arachidonic acid (200 μM). B, the addition of 1 μM GTP[γS] (trace 1), 100 μM ATP[γS] (trace 2) or 100 μM ATP[γS] plus 1 mM UDP (trace 3). C, the addition of 1 μM GTP[γS]. D, the addition of 100 μM ATP[γS] (trace 1) or 1 μM GTP[γS] (trace 2). The intersections of the dashed lines with trace 3 represent the lag periods required for the reinitiation of O_2^- formation, defined from extrapolation of the linear part of the absorbance curve to basal absorption values. Superimposed typical tracings of parallel experiments are shown. Similar results were obtained in at least three independent experiments
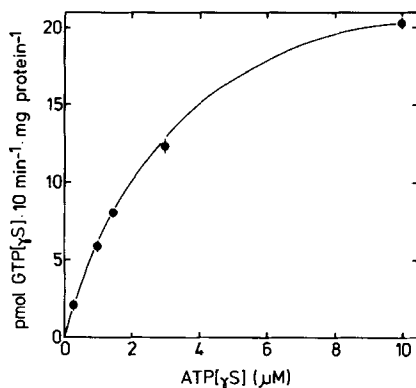

Fig. 4. *Formation of [8-³H]GTP[γS] from ATP[γS] and [8-³H]GDP by membranes of HL-60 cells.* Formation of [8-³H]GTP[γS] by membranes of HL-60 cells was determined as described in Materials and Methods in the presence of 0.5 μM [8-³H]GDP and the indicated concentrations of ATP[γS]. Data are the mean ± SD of three experiments
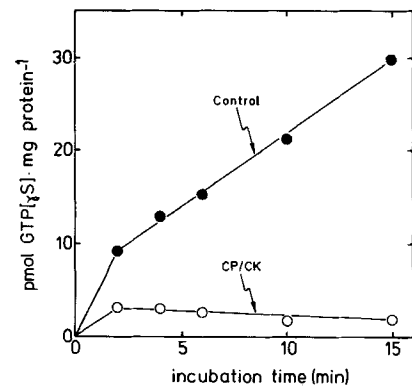

Fig. 5. *Time course of [8-³H]GTP[γS] formation from ATP[γS] and [8-³H]GDP by membranes of HL-60 cells: influence of a NTP-regenerating system.* The formation of [8-³H]GTP[γS] was determined in the presence of 0.5 μM [8-³H]GDP and 50 μM ATP[γS] for the indicated periods of time with (○) or without (●) 20 μg creatine kinase plus 5 mM creatine phosphate. Data are the mean of assay duplicates

Finally we measured the formation of GTP[γS] from ATP[γS] and GDP. As shown in Fig. 4, formation of GTP[γS] in HL-60 membranes depended on the concentration of ATP[γS]. Half-maximal GTP[γS] formation was observed at an ATP[γS] concentration of 3−4 μM. The formation of GTP[γS] did not occur in the absence of Mg^{2+} (data not shown). Time course experiments revealed that the initial rapid rate of GTP[γS] formation for the first 2 min declined thereafter to a lower but still substantial rate (Fig. 5). The presence of CK/CP almost completely blocked the formation of GTP[γS]. There was still some formation of GTP[γS] in the very early phase of the incubation, which, however, completely ceased thereafter. The accumulation of [8-³H]GTP[γS] in the presence of CK/CP decreased slightly with increasing time of incubation, suggesting that unlabelled GTP[γS] was formed from [8-³H]GTP[γS]. As shown in Table 2, more [8-³H]GTP[γS] was formed with GTP[γS] than with ATP[γS] (each 50 μM) together with 0.5 μM [8-³H]GDP as thiophosphoryl group acceptor. In contrast to the [8-³H]GTP[γS]

formation by ATP[γS], that induced by GTP[γS] was only reduced by about 30% in the presence of CK/CP.

DISCUSSION

Evidence is presented that NDPK reactions play a role in the G protein activation of NADPH oxidase by ATP[γS]. First, in the absence of Mg^{2+}, which is essential for NDPK activity [14], there was no formation of GTP[γS] and also no functional response to ATP[γS]. In addition, the concentrations of ATP[γS] required for half-maximal NADPH oxidase activation and GTP[γS] formation were very similar. Furthermore, in the presence of a tenfold higher concentration of ATP in comparison with ATP[γS] O_2^- formation was suppressed. This finding may be explained by competition of these nucleotides for transphosphorylation. ATP leads to the formation of weakly active GTP and prevents formation of strongly stimulatory GTP[γS] by ATP[γS]. Interestingly all agents which inhibited NDPK activity reduced basal NADPH

oxidase activity by 30−40%. ATP enhanced basal O_2^- formation by 36%, probably by formation of GTP. Additionally the removal of ATP from assay mixtures by preincubation with hexokinase and glucose reduced arachidonic-acid-induced O_2^- formation by more than 50% [9]. Thus, there may be continuous formation of GTP by NDPK, using ATP as phosphoryl group donor, leading to basal stimulation of the G protein and of NADPH oxidase. In the presence of NDP, forming an abortive NDP-NDPK complex [14, 15, 18, 25], the stimulatory effect of ATP[γS] was prevented. The involvement of NDPK reactions in NADPH oxidase activation by ATP[γS] is also supported by the finding that the lag periods required for the activation of NADPH oxidase by ATP[γS] were about two times longer than those observed with GTP[γS]. In comparison to NDPK, ATP[γS] is a poor substrate for protein kinase C [27]. In addition, protein kinase C is inhibited by ADP at concentrations as low as 5 μM [27], whereas NDP inhibited O_2^- generation only at a concentration of 1 mM. These results support the view that protein kinase C is not involved in the activation of NADPH oxidase by arachidonic acid in cell-free systems [2, 8, 9, 12].

The stimulatory effects of ATP[γS] and ATP on NADPH oxidase did not depend on the presence of added GDP. GDP may be present at sufficiently high concentrations as an endogenous component in the membrane and/or cytosol. Evidence for the role endogenous GDP in the formation of GTP[γS] by NDPK was obtained by removing endogenous NDPs by CK/CP. In the presence of CK/CP, the stimulatory effect of ATP[γS] on NADPH oxidase and the formation of GTP[γS] were suppressed. These data indicate that the GDP used for the formation of GTP[γS] is not tightly bound to G proteins [13, 28 − 30] but is loosely associated with some component of the membrane or cytosol and, hence, is accessible for CK.

The mechanism by which GTP[γS] stimulates NADPH oxidase appears to be complex: the stimulatory effect of GTP[γS] was considerably reduced but not abolished in the presence of EDTA, suggesting that for G protein activation by GTP[γS] still enough Mg^{2+} was present and/or that Mg^{2+} was only slowly removed from the G protein by the EDTA treatment [31]. However, as NDPK activity also depends on the presence of Mg^{2+} [14], the chelation experiments did not rule out a role of this kinase in NADPH oxidase activation by GTP[γS]. The NTP-regenerating system depleted GDP pools and concomitantly led to the formation of GTP, the latter nucleotide only weakly enhancing O_2^- formation. Thus, the stimulatory action of GTP[γS] was considerably reduced by CK/CP. GTP[γS] was a better substrate than ATP[γS] for GTP[γS] formation. In contrast to the GTP[γS] formation with ATP[γS] as substrate, that observed with GTP[γS] as substrate was only partially inhibited by GDP removal, suggesting that the GDP, which is used for the GTP[γS] formation with GTP[γS], is only partially accessible to CK. This pool of GDP may, thus, is analogy to other G proteins be tightly bound to G proteins in HL-60 membranes and serve as a thiophosphoryl group acceptor [13, 8 − 30]. However, further investigation is required to identify different pools of GDP serving as phosphate acceptors. Interestingly, NDPK has been shown to catalyze the transfer of phosphate groups to GDP bound to various G proteins [16, 20, 21]. We recently observed that the complete removal of endogenous and exogenous ATP enhanced NADPH oxidase stimulation by GTP[γS] [9], suggesting that competition of ATP and GTP[γS] for phosphoryl/thiophosphoryl group transfer to GDP was eliminated. Therefore, transthiophosphorylation reactions may also take part, at least to some extent, in the G protein activation by GTP[γS]. The NDPK reaction involved in the activation of NADPH oxidase by GTP[γS] is resistent to inhibition by ADP and UDP at the concentrations used, suggesting that various species of NDPK may be involved in the process of G protein activation.

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