Nucleoside Triphosphate Requirements for Superoxide Generation and Phosphorylation in a Cell-free System from Human Neutrophils

SODIUM DODECYL SULFATE AND DIACYLGLYCEROL ACTIVATE INDEPENDENTLY OF PROTEIN KINASE C*

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The NADPH-oxidase of human neutrophils can be activated in a cell-free system comprised of plasma membrane, cytosol, and an anionic amiphile such as arachidonate or sodium dodecyl sulfate (SDS). Recently, we showed that dicylglycerol acts synergistically with SDS in the cell-free system to stimulate superoxide generation, with concurrent phosphorylation of a 47-kDa cytosolic protein which is thought to be a component of the oxidase (Burnham, D. N., Uhlinger, D. J., and Lambeth, J. D. (1990) J. Biol. Chem. 265, 17550-17556). We report herein that when undialyzed cytosol is used along with either SDS alone or SDS plus dicylglycerol as activators, adenosine 5'-[(γ-thio)triphosphate (ATPγS) and guanosine 5'-[(γ-thio)triphosphate (GTPγS) both stimulated superoxide generation several fold, yielding about the same maximal velocity. ATP and GTP showed lower levels of stimulation. Stimulation by ATPγS and GTPγS was nonadditive, and showed a 5-7-fold greater specificity for GTPγS. ATPγS stimulation was inhibited by the nucleoside diphosphate (NDP) kinase inhibitor UDP. In contrast, when extensively dialyzed cytosol was used, most of the stimulation by ATPγS was lost, while most of that by GTPγS was retained. Addition of GDP restored the ability of ATPγS to stimulate, consistent with NDP kinase-catalyzed formation of GTPγS from ATPγS plus GDP. This activity was demonstrated directly in both cytosol and plasma membrane. Using undialyzed cytosol, phosphorylation of p47 showed a similar nonspecificity for nucleoside triphosphates, due to NDP kinase activity, but revealed the expected ATP specificity when dialyzed cytosol was used. Neither ATPγS nor GTPγS were good substrates for protein phosphorylation. Under a variety of conditions, phosphorylation of p47 or other neutrophil proteins failed to correlate with oxidase activation. The present studies indicate that SDS and dicylglycerol stimulation of superoxide generation in the cell-free system is independent of protein kinase C or other protein kinase activity, and suggest a novel role for dicylglycerol in cell regulation.

The NADPH-dependent respiratory burst oxidase (NADPH-oxidase) of human polymorphonuclear leukocytes provides the critical cellular defense against invading microorganisms. The oxidase exists in a dormant state in unstimulated neutrophils, but can be activated by a variety of stimuli, including the chemoattractant formyl-methionyl-leucyl-phenylalanine, protein kinase C activators such as phorbol 12-myristate 13-acetate, and particulates such as opsonized bacteria or zymosan (see Ref. 1 for review). The activated oxidase catalyzes the univalent reduction of molecular oxygen to superoxide which secondarily generates other reactive oxygen species (e.g. H2O2, HOCl, OH) which participate in oxidative killing of ingested microbes (2). The importance of the NADPH-oxidase is illustrated by the inherited condition chronic granulomatous disease, a family of biochemical diseases wherein defects in one of several proteins of the oxidase result in an inability to activate the respiratory burst, with a consequent impairment of affected individuals to combat infections (3). Identified by their absence in variants of chronic granulomatous disease, protein components of the respiratory burst oxidase (or related to its activation) include a plasma membrane-bound heterodimeric hemoprotein, cytochrome b558 (25- and 92-kDa subunits) (4, 5), and two cytosolic components, p47phox and p67phox (6, 7). The latter translocate to the plasma membrane upon activation, and fail to do so in chronic granulocytomous disease variants lacking cytochrome b558, suggesting that these components participate in a complex with the cytochrome (8-10).

A role for phosphorylation in activation in intact cells has been proposed, based on the ability of protein kinase C agonists to activate the respiratory burst (for reviews, see Ref. 1). A potential mechanism for activation involves the phosphorylation of p47phox, which occurs in parallel with activation by both phorbol 12-myristate 13-acetate and formyl-methionyl-leucyl-phenylalanine (11-14). Recent studies using intact cells have demonstrated multiple phosphorylations of this protein (13, 14), some of which require association with the cytochrome (15). Nevertheless, other studies (16, 17) have noted a lack of correspondence between phosphorylation and activation under certain conditions, and a causal role for phosphorylation in activation remains unclear. Indeed, a role for phosphorylation in down-regulation is possible, and specific phosphorylations of p47phox might either activate or inhibit, depending upon site-specific phosphorylation.

Because of its extreme lability (18), the activated oxidase has been difficult to characterize in broken cell preparations from activated neutrophils. However, a cell-free activation system has recently been described (19-21), and consists of plasma membrane and cytosol, both from unstimulated neutrophils, along with an anionic amiphile such as SDS1 or

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1 The abbreviations used are: SDS, sodium dodecyl sulfate; diC8, 1,2-di-Octanoylglycerol; PIPES, 1,4-piperazinebisethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitriilo)tetraacetic acid; ATPγS,
Nucleotide Requirements for NADPH-oxidase

Materials—Hespan (6.2% hetastarch in 0.9% NaCl) was obtained from American Hospital Supply Corp. Lymphocyte separation medium (6.2% Ficoll, 9.4% sodium diatrizoate) was purchased from Bionetics Laboratory Products. 1,2-Diacylglycerol was purchased from Avanti Polar Lipids (Birmingham, AL). NADPH, ATP, ADP, GTP, GDP, cytochrome c (type IV; horse heart), and diisopropylfluorophosphate were obtained from Sigma. ATP-S and GTP-S were purchased from Boehringer Mannheim. Sodium dodecyl sulfate was obtained from Bio-Rad. Polyethyleneimine-cellulose thin-layer chromatography sheets, containing a fluorescent indicator, were from EM Science. [γ-32P]ATP (4000 Ci/mmole), [γ-32P]GTP (1500 Ci/mmole), and [8-3H]GDP (10 Ci/mmole) were from Amersham.

Isolation of Human Neutrophils—Human neutrophils were isolated from peripheral blood from healthy adult donors as described previously (41). Informed consent was obtained from all subjects. Isolated cells were suspended in phosphate-buffered saline (2.6 mM KC1, 1.5 mM KH2PO4, 0.5 mM MgCl2, 136 mM NaCl, 8 mM NaHCO3, 0.6 mM CaCl2) containing 5.5 mM glucose and were subsequently incubated with 4 mM diisopropylfluorophosphate for 30 min at 37°C.

Preparation of Plasma Membrane and Cytosolic Fractions—Neutrophils (1-1.5 x 107 cells) were suspended in 10 ml of phosphate-buffered saline-glucose and incubated on ice with 4 mM diisopropylfluorophosphate for 30 min. The cells were pelleted at 900 x g for 7 min at 4°C and were resuspended in incubation buffer A (100 mM KCl, 3 mM NaCl, 4 mM MgCl2, and 10 mM PIPES, pH 7.0, containing 1 mM EGTA, 2 μM leupeptin, 2 μM pepstatin, and 0.5 mM phenylmethylsulfonyl fluoride). The cells were adjusted to 1.0-1.5 x 107 cells/ml with the same buffer and were disrupted by nitrogen cavitation after being pressurized at 450 p.s.i. for 25 min at 4°C (17). The cell suspension was centrifuged at 600 x g for 1 h at 4°C to remove any residual cell material. The plasma membrane fraction was concentrated to 10-15 mg/ml using Centricon (Amicon) filtration devices equipped with 10-kDa molecular mass cut-off filters. Both the membrane and cytosol fractions were stored in aliquots (50-150 μl) at -80°C. Samples stored in this manner were stable for at least 3 months. Extensively dialyzed cytosol was prepared by dialysis (12-14 KDa cut-off) at 4°C against a 2000-fold excess volume of buffer A, with three changes of buffer at -6 h intervals.

Incubations—Reaction mixtures contained 20 μg of plasma membrane, 100 μg of cytosol, and varying amounts of agonists (e.g. nucleotide triphosphates, SDS, and diC8) in a total volume of 50 μl. Three 10-μl aliquots of each reaction mixture were transferred to six-well assay plates (Corning) and preincubated for 5 min at 25°C. The preincubation step prior to assay permits activation in the presence of concentrated components, allowing conservation of materials and high activities, as described previously (17). At the end of the preincubation period, NADPH (200 μl), cytochrome c (80 μM), and 240 μl of buffer A were added to initiate superoxide generation.

Superoxide Generation—Superoxide was quantified at 25°C by monitoring cytochrome c reduction using a thermostatted Molecular Devices Thermomax Kinetic microplate reader equipped with a 590-nm wavelength filter with a 1-nm bandwidth, as described (17). In parallel samples, superoxide dismutase (10 U/ml) was added after approximately 30 s to inhibit superoxide-dependent cytochrome c reduction. An extinction coefficient of 550 nm of 21 mMcm⁻¹ was used to calculate the quantity of cytochrome c reduced (42). The rate of cytochrome c reduction in the presence of superoxide dismutase was subtracted from that in the absence of the enzyme to determine superoxide-mediated cytochrome c reduction. For each preparation of cytosol and membranes, the optimal concentration of SDS for activation was

plained by the presence of a nucleoside diphosphate kinase activity in cytosol and plasma membrane. The data indicate that diacylglycerol/SDS costimulation of the respiratory burst oxidase is not protein kinase-mediated, and suggest a novel, protein kinase C-independent role for diacylglycerol in cell activation.

EXPERIMENTAL PROCEDURES

The nucleoside triphosphate and metal ion requirements for oxidase activation have been examined, both in electro-permeabilized neutrophils and in the cell-free activation system. Data from permeabilized cells indicate that both ATP and GTP participate in activation (23-25), and pharmacological approaches in this system implicate a protein kinase, perhaps protein kinase C (25). Nevertheless, in permeabilized cells, it is not clear whether the nucleoside triphosphate requirements relate to the oxidase itself, or to upstream signal transduction components. In the cell-free system, which may reflect only the down-stream oxidative components, there is a clearly magnesium requirement. In addition, there is general agreement that guanine nucleotide analogs such as GTP-γS and Gpp(NH)p stimulate superoxide generation by 2-4-fold above that on short-chain diradylglycerols alone (26-29). This, together with the observation that fluorode augment activity, has lead to the proposal of an oxidase-linked guanine nucleotide regulatory protein (G protein). Data regarding an adenosine triphosphate requirement are less clear. Several groups (26, 28, 30, 31) report no requirement for ATP or ATP analogs, while others (29, 32) have noted up to a 2-fold stimulation by adenosine triphosphate or its thophosphoryl analog ATP-γS. In some cases, interpretations may have been affected by the presence of endogenous nucleotides in isolated cytols (33). Thus, while GTP analogs clearly augment activation, the participation of adenosine triphosphate seems less clear.

We (34-37) and others (38-40) have shown that under a variety of activation conditions, diacylglycerol generation correlates with superoxide generation. Because of previous data implicating the involvement of both protein kinase C and p47phox phosphorylation in this process, we recently investigated the effects of diacylglycerol and other protein kinase C activators on superoxide generation in the cell-free system (17). We discovered that although they are ineffective alone, short-chain diradylglycerols synergize with the anionic amphiphile to augment oxidative activities from 2.5 to 7-fold. In these studies, diacylglycerol also augmented phosphorylation of p47phox. Although it was tempting to speculate that protein kinase C was involved in the synergistic activation, several features of activation (e.g. calcium requirements, diacylglycerol specificity, lack of effect of inhibitors) did not appear to be consistent with a role for classical protein kinase C isoforms.2 In the present studies, we have undertaken the examination of the nucleoside triphosphate (NTP) requirements for activation and phosphorylation in the cell-free system, using SDS, both alone and in combination with diC8. We find that for both activation conditions, the actual nucleotide triphosphate (NTP) requirement is 5'-gγ-thiotriphosphate; App(NH)p, adenyI-5'-yl β,γ-imidodiphosphate; GTP-γS, guanosine 5'-O-(γ-thiotriphosphate); Gpp(NH)p, guanosine 5'-O-(γ-thioimidophosphate); NDP kinase, nucleoside diphosphate kinase.

For example, diacylglycerol analogs (e.g., 1,3-diacylglycerol, 1-O-alkyl-2-acetylglcerol) which are not known to activate protein kinase C are partially effective in the cell-free system. A variety of other diacylglycerol analogs and detergents, however, were ineffective, indicating that the diacylglycerol effect is structurally specific.

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determined prior to carrying out experiments. This concentration varied from 150 to 35 μM, depending on the preparation.

**Protein Phosphorylation**—Reaction mixtures containing cytosol, plasma membrane, and various effectors were as described above. The 45-μg mixtures were kept on ice until the reaction was initiated with 5 μl of 2.7 mM γ-[32P]ATP or [γ-32P]GTP (approximately 1 μCi of label added, 270 μM nucleotide final concentration). For some experiments, [γ-32P]ATP or [γ-32P]GTP were used at the same concentration and specific activity. The mixture was incubated for 10 min at 25 °C with shaking, and 50 μl of 2 × SDS Laemmli sample buffer was then added (49). Samples were heated at 100 °C for 3 min and centrifuged briefly prior to loading onto 12.5% gels for SDS-polyacrylamide gel electrophoresis.

**Polyacrylamide Gel Electrophoresis and Autoradiography**—Samples, approximately 12 μg of protein per lane, were subjected to electrophoresis on minigels (6 cm × 10 cm × 1 mm) using a modified Laemmli system (43). The running gel and stacking gel consisted of 12.5 and 5% acrylamide, respectively, with bis-acrylamide/acrylamide ratios of 1:180 and 1:37.5, respectively. After staining with Coomassie Blue, the gels were destained and dried onto Whatman 3 filter paper. The dried gels were subjected to autoradiography on Kodak X-Omat AR film overnight at ~80 °C, with an intensifying screen. For "8-

**Nucleotide diphosphorylase Kinase Activity**—Reaction mixtures (45 μl) contained 40 μg of plasma membrane and/or 200 μg of either native or dialyzed cytosol, 4 μM [γ-32P]GDP (approximately 2 μCi per reaction), and buffer A. ATPyS (5 μl of 1 mM) was added to initiate the reaction, and the mixture was incubated for 10 min at 25 °C with shaking. The reaction was terminated by the addition of EDTA to a final concentration of 17 mM. Samples were centrifuged for 15 min at 4,000 × g, and a 10-μl aliquot of the supernatant was mixed with 0.5 μl of carrier unlabeled GTP, GDP, and GMP (each at 1 mM final concentration) and applied to polyethyleneimine-cellulose thin-layer chromatography plates. The plates were developed in 0.75 M KH2PO4 at room temperature as described (29). The nucleotides were visualized by fluorescence at 254 nm and the areas corresponding to standards were scraped and eluted for liquid scintillation counting.

**Data Analysis**—The maximal rates of cytochrome c reduction from triplicate aliquots from each reaction mixture were averaged to obtain a single rate value for each incubation. A minimum of three separate incubations were then averaged to provide standard error information. The data presented graphically are representative of results obtained from three preparations of membranes and cytosol obtained from separate donors. EC50 and Vmax values were determined using a nonlinear regression data analysis as described previously (44).

**RESULTS**

**Effect of Nucleoside Triphosphates on Superoxide Generation in the Cell-free System**—Results using plasma membrane plus cytosol, activated with SDS, are shown in the two left panels of Fig. 1. In the absence of added nucleotide, a basal activity of 250–300 nmol of cytochrome c reduced/min/mg plasma membrane protein was seen. ATP (100 μM) augmented the activity slightly less than 2-fold (Fig 1, upper left panel, filled circles), and there was no further stimulation by concentrations up to 400 μM (not shown). A larger (2.5-fold) augmentation which occurred at lower concentrations (optimum at 20 μM) was seen with ATPyS (Fig. 1, upper left panel, open circles). Using SDS alone, GTPyS had little or no stimulatory effect at levels up to 100 μM (Fig. 1, lower left panel, filled squares). GTPyS, however, augmented oxidase activity (open squares) to a similar degree as ATPyS.

The activating effects of nucleoside triphosphates were also seen when an optimal concentration of diC8 was included along with SDS in the preincubation (Fig. 1, right panels). diC8 alone augmented the basal rate by more than 2-fold to 544 nmol/min/mg plasma membrane protein. The upper panel shows the effects of the adenine nucleotides. ATP at 50 μM and higher concentrations increased the rate 1.6-fold (upper right, filled circles). ATPyS resulted in a larger (2.6-fold) enhancement of oxidase activity to more than 1400 nmol/min/mg plasma membrane protein (upper right, open circles). The lower right panel shows the effects of guanine nucleotides, using diC8 along with SDS to activate. There was a modest increase with GTP alone (1.5-fold at 100 μM) but a larger (3.3-fold) effect was seen with GTPyS (10 μM), which resulted in a rate of more than 1500 nmol/min/mg plasma membrane protein. Thus, the presence of diacylglycerol increases both basal and nucleoside triphosphate–augmented activities.

**Data from the right panels in Fig. 1 using the optimal activators ATPyS and GTPyS were replotted in Fig. 2 in a Lineweaver-Burk format after subtraction of basal activities.**
TABLE I

Effect of ATPγS and GTPγS on the kinetics of NADPH-oxidase activity in cell-free systems

Preincubation and assay conditions were as in Figs. 1 and 4, and "Experimental Procedures." To permit a good kinetic fit to the data, rates were initially corrected by subtracting the basal activity (i.e., that seen with no added nucleotide) prior to determination of kinetic parameters by a nonlinear least-squares method, as under "Experimental Procedures." The $E_{\text{max}}$ value shown represents the sum of the basal activity and the extrapolated maximal stimulation by nucleotide. The basal rates in nmol cytochrome $c$ reduced/min/mg plasma membrane (PM) protein were as follows: in the native (undialyzed system) the rate with SDS alone was 245, and 544 with SDS plus diC8, and with dialyzed cytosol the rate with SDS was 56, and that with SDS plus diC8 was 155.

| Cell-free system/agonist | Nucleotide added | EC$_{50}$ $\mu M$ | $V_{\text{max}}$ nmol cyt. $c$ red./min/mg PM protein |
|--------------------------|-----------------|------------------|--------------------------------------------------|
| Native SDS               | ATPγS           | 4.8              | 676                                              |
|                          | GTPγS           | 1.0              | 868                                              |
|                          | SDS + diC8 ATPγS| 7.7              | 1483                                             |
|                          | GTPγS           | 1.1              | 1596                                             |
| Dialyzed SDS             | ATPγS           | N/A$^*$ (56)     |                                                  |
|                          | GTPγS           | 1.8              | 620                                              |
|                          | SDS + diC8 ATPγS| 12.5             | 410                                              |
|                          | GTPγS           | 1.4              | 1355                                             |

$^*$ Stimulation was too low to accurately determine kinetic parameters. The $V_{\text{max}}$ in this case represents the basal rate.

![Fig. 3. Effects of combinations of ATPγS and GTPγS on superoxide generation in the cell-free system.](image)

EC$_{50}$ and $V_{\text{max}}$ values are summarized in Table I, under "SDS + diC8." The EC$_{50}$ for GTPγS was 1.1 $\mu M$, 7-fold lower than that for ATPγS. The $V_{\text{max}}$ values using both thiophosphoryl nucleotides were essentially identical. Also shown in Table I are EC$_{50}$ and $V_{\text{max}}$ values for ATPγS and GTPγS stimulation using SDS alone as activator. EC$_{50}$ values for both nucleoside triphosphates were the same within experimental error as those seen when diC8 was included, again demonstrating a preference for the guanosine triphosphate analog. $V_{\text{max}}$ values were about half of those seen when diC8 was included, and showed a slight preference for the guanine nucleotide. Thus, both nucleotides activated superoxide generation to an approximately equal extent, and stimulation was more specific for GTPγS than ATPγS.

The similarity in $V_{\text{max}}$ values suggested that ATPγS and GTPγS might be activating by the same mechanism. If this were the case, then simultaneous addition of both nucleotides should be nonadditive. Results using combinations of the two nucleotides are summarized in Fig. 3. The concentrations of nucleotides utilized were those that elicited the maximal responses in Fig. 1. As shown in Fig. 3, there was no additivity observed when SDS alone was used to activate. Although there was a slight additional activation seen by the combined nucleotides when diC8 was included, the rate was considerably less (~1750 nmol/min/mg plasma membrane protein) than that which would be predicted for additive rates (~2400 nmol/min/mg plasma membrane protein). Thus, ATPγS and GTPγS do not stimulate in an additive manner, suggesting that they may act by the same mechanism.

**Effect of Dialyzed Cytosol on the Nucleoside Triphosphate Dependence of Superoxide Generation in the Cell-free System**—Because nucleotides are known to be present in neutrophil cytosol (33) and might complicate the interpretation of nucleotide-dependent activation, we repeated the experiments shown in Fig. 1 using extensively dialyzed cytosol. Basal activities were 4–6-fold lower using dialyzed cytosol, both with SDS and with SDS plus diC8 as activators, consistent with the loss of low molecular weight stimulatory factor(s). With SDS alone (Fig. 4, upper left panel), neither ATP (filled circles) nor ATPγS (open circles) activated to an appreciable degree. Results with guanine nucleotides, however, were essentially the same as those obtained with undialyzed cytosol, although the maximal activity achieved was somewhat lower. GTPγS (10 $\mu M$) stimulated oxidase activity about 10-fold, from a basal rate of 56 nmol of cytochrome $c$ reduced/min/mg plasma membrane protein. The degree of stimulation was higher in the dialyzed system (9–2.5-fold), primarily due to a lower basal activity. Presumably, the presence of endogenous nucleoside triphosphates in the undialyzed cytosol elevates the basal rate significantly.

When diC8 was used along with SDS in the dialyzed system (upper right panel, Fig. 4), there was a very modest stimulation of activity by ATP and a slightly greater effect of ATPγS, but this response remained much less than that observed when native cytosol was used (compare with Fig. 1). As shown in the lower right panel of Fig. 4, GTP again had only a small (40%) stimulatory effect (filled squares). However, as in the
Undialyzed system, GTPγS continued to produce a large stimulatory effect (open squares), and achieved a level of stimulation almost as great as that obtained when undialyzed cytosol was used (compare lower right panels of Figs. 1 and 4). As summarized in Table I, the EC50 and Vmax values for GTPγS were relatively unchanged by dialysis. However, the Vmax for ATPγS stimulation was about 6-fold less when dialyzed cytosol was used. Thus the removal of soluble low molecular weight components by dialysis drastically reduces the ability of adenine nucleotides to stimulate in the cell-free system, but activation by guanine nucleotides remains largely intact.

GDP Restores the Ability of ATPγS to Stimulate Activity Using Dialyzed Cytosol, and UDP Inhibits ATPγS Stimulation—A possible explanation for the apparent nonspecificity for nucleotides in the native cytosol is that NDP kinase has catalyzed the transfer of the thiophosphoryl groups among nucleotides, e.g., from ATPγS to endogenous GDP to form GTPγS. Thus when, dialyzed cytosol is used, there is no longer free GDP to act as substrate acceptor for the (thio)phosphoryl group. To test this hypothesis we used the cell-free system consisting of plasma membrane and dialyzed cytosol, as in Fig. 4. Results with SDS plus dC8 are presented in Fig. 5. GTPγS (open squares) again stimulated activity markedly (7.8-fold), and showed an optimum at 10 μM. As in Fig. 4, there was only a modest stimulatory effect of ATPγS alone (open circles, Fig. 5). However, inclusion of 10 μM GDP along with ATPγS restored the ability of the ATPγS to activate (filled triangles), and rates near those seen with GTPγS were observed. The concentration of GDP used was critical to observe restoration of activity by ATPγS, since 2-fold higher or lower concentrations resulted in a much smaller degree of stimulation (data not shown). These data support the idea that the loss of ATPγS stimulation upon dialysis of cytosol is due at least in large part to removal of GDP, which is functioning as a thionphosphoryl acceptor. Thus, following dialysis, the actual nucleoside triphosphate dependence of the system is revealed. In support of this interpretation, UDP, which can function as an inhibitor of NDP kinase, inhibited ATP and ATPγS but not GTPγS stimulation of oxidase activity (IC50 = 0.75 mM using 200 μM ATP or ATPγS; 350 μM SDS and 200 μM dC8) when undialyzed cytosol was used (data not shown), confirming results by Seifert et al. (29) using arachidonate.

![Image](image-url)

**Fig. 5.** Effect of GDP on recovery of ATPγS stimulation of superoxide generation in the cell-free system. Preincubations and treatment groups were as described in Fig. 1, except that extensively dialyzed cytosol was used in place of native cytosol. Each incubation included both SDS (250 μM) and dC8 (200 μM), and where indicated (closed triangles) GDP (10 μM). Data points represent the mean ± S.E. (n = 6) from one preparation. Where not shown, the error bar did not exceed the size of the symbol. Similar results were obtained using preparations of cytosol and plasma membrane from three separate donors.

### Table II

| Component added | [3H]GTPγS pmol |
|-----------------|---------------|
| None            | 1.9 ± 0.5a    |
| PM              | 79.6 ± 1.8    |
| CYT             | 195.4 ± 3.4   |
| CYT + PM        | 122.2 ± 4.3   |
| DCYT            | 216.4 ± 3.8   |
| DCYT + PM       | 154.9 ± 0.6   |

*Standard error of three incubations.

**Nucleoside-diphosphate Kinase Activity in Plasma Membrane and Cytosol**—To test directly whether NDP kinase activity in cytosol and plasma membrane preparations could account for the observed nucleotide specificity, we examined the ability of these fractions to catalyze thionphosphoryl transfer from ATPγS to [3H]GTP. Results are shown in Table II. NDP kinase activity was present in both cytosol and plasma membrane, although the activity in the combined fractions was somewhat lower than that expected from the individual activities. The activity was higher when dialyzed cytosol was used, presumably due to dilution of the tritium label in the undialyzed sample with unlabeled GDP and/or other nucleotides. Neither SDS nor SPS plus dC8 at concentrations used in the superoxide assays affected the nucleoside-diphosphate kinase activity (data not shown).

**Nucleoside Triphosphate Dependence for Phosphorylation of p47**

Using Undialyzed and Dialyzed Cytosol—Both [γ-32P]ATP and [γ-32P]GTP were used as phosphoryl donors under activation conditions given in Figs. 1 and 4, and phosphorylation was monitored by SDS-polyacrylamide gel electrophoresis followed by autoradiography, and under "Experimental Procedures." As shown in Fig. 6A, when native (undialyzed) cytosol was used, both SDS (S) and diacylglycerol (D) alone and in combination (S/D) enhanced the phosphorylation of both a 47-kDa protein and a 68-kDa protein. [γ-32P]ATP and [γ-32P]GTP functioned equally well as phosphoryl donors under these conditions. Comparison of Figs. 1 and 6 reveals that phosphorylation failed to correlate with activation. For example, with SDS alone or combined with diacylglycerol, ATP and GTP served equally as phosphoryl donors to protein, but only ATP activated to a significant extent. Diacylglycerol alone promoted heavy phosphorylation with either nucleotide, but in the absence of SDS fails to activate superoxide generation (17). With dialyzed cytosol (Fig. 6, lower panel), GST lost much of its ability to serve as a phosphoryl donor to protein, while ATP-dependent p47 phosphorylation remained largely intact. Phosphorylation of the p47 again failed to correlate with activation (compare Fig. 6B with Fig. 4). Using all combinations of activators, ATP remained a good phosphoryl donor to p47, but lost most of

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1. It should be noted that an apparently synergistic effect of diacylglycerol and SPS on p47 phosphorylation was seen previously by us (17), but conditions (tracer concentration of added [γ-32P]ATP and shorter reaction time) differed considerably from those of the present studies. In the present studies which use more physiological concentrations of ATP and GTP, we found a moderate stimulation of p47 phosphorylation by SPS, a generally higher activation by diacylglycerol alone, and little or no additive or synergistic effect of the two together. Densitometry was carried out for all experiments and confirmed the visual impression.
its effectiveness in supporting superoxide generation. Thus, ATP rather than GTP is the primary phosphoryl donor for protein phosphorylation, but phosphorylation fails to correlate with activation.

In an experiment analogous to that shown in Fig. 5, ADP was included along with \( \gamma^{-32}\text{P}\)GTP in the system utilizing dialyzed cytosol (Fig. 6B). Under these conditions, phosphorylation of p47 by \( \gamma^{-32}\text{P}\)GTP was restored to a level near that seen using undialyzed cytosol. Thus, the non-specificity for nucleotide triphosphate-dependent protein phosphorylation in the system using undialyzed cytosol was due to nucleoside-diphosphate kinase-dependent transfer of the \( \gamma^{-32}\text{P}\)phosphate from \( \gamma^{-32}\text{P}\)GTP to ADP to form \( \gamma^{-32}\text{P}\)ATP, which was then utilized for protein phosphorylation. To address the possibility that the large activations seen with ATP\(\gamma\text{S}\) and GTP\(\gamma\text{S}\) were the result of (thio)phosphorylations catalyzed by an unknown kinase, experiments exactly analogous to those presented in Fig. 6 were performed except that \( \gamma^{-32}\text{S}\)ATP\(\gamma\text{S}\) and \( \gamma^{-32}\text{S}\)GTP\(\gamma\text{S}\) were used as thosphoryl donors. Virtually no label was incorporated using either nucleotide with SDS or diC8, alone or in combination.

**DISCUSSION**

We recently described (17) the stimulatory effect of diacylglycerol on superoxide generation in a cell-free system consisting of plasma membrane plus either native or dialyzed cytosol. Incubations, SDS-polyacrylamide gel electrophoresis, and autoradiography were as detailed in Fig. 1 and under "Experimental Procedures." A, autoradiogram obtained from SDS-polyacrylamide gel electrophoresis-resolved phosphoproteins derived from incubations containing native (undialyzed) cytosol while \( B \) is that using extensively dialyzed cytosol, both in addition to plasma membrane. Parallel incubations were carried out using 270 \( \mu\text{M} \) of either ATP \(( A)\) or GTP \(( G)\), and 10 \( \mu\text{M} \) ADP was included as indicated. Incubations contained either no activator \((-\)), 350 \( \mu\text{M} \) SDS \(( S)\), 200 \( \mu\text{M} \) diC8 \(( D)\), or SDS plus diC8 \(( S/D)\). The arrow corresponds to a protein migrating with an apparent molecular weight of 47,000. The results shown are representative of two experiments done with different cytosol and plasma membrane preparations.

We have repeatedly observed that the stimulation of superoxide generation by diacylglycerol would require ATP. Tauber and colleagues (45, 46) had previously described activation of superoxide generation by phorbol esters in a cell-free system in which the cytosolic requirement could be replaced by purified protein kinase C and showed an ATP requirement in this system. Nevertheless, the activity achieved was very low (less than 0.7% of that seen in the presented studies), and we (17) and others (47) have repeatedly failed to observe significant activation by phorbol esters, either in the presence of cytosol or purified protein kinase C. In the face of conflicting reports with regard to an ATP requirement in the standard cell-free system \((i.e.\, without\, diacylglycerol)\), we undertook the present studies to determine under our assay conditions the NTP requirements with SDS alone, and to define the NTP requirements for the diacylglycerol-linked stimulation.

As shown in Figs. 1 and 4, qualitatively similar NTP requirements were seen regardless of whether SDS alone or SDS plus diacylglycerol were used as activators. In the undialyzed cytosol (Fig. 1), ATP\(\gamma\text{S}\) and GTP\(\gamma\text{S}\) were both effective in stimulating superoxide generation under both activation conditions. ATP was somewhat effective, while GTP was the least effective, and in the case of SDS activation, did not activate at all. Diacylglycerol did not alter the NTP specificity; rather its effect was to increase the maximal velocity at optimal NTP concentrations. In the undialyzed system, the similarity of \( V_{\text{max}} \) values using both ATP\(\gamma\text{S}\) and GTP\(\gamma\text{S}\) as well as their nonadditive effects were consistent with a common stimulatory mechanism. The lower \( E_{\text{Ca}} \) for GTP\(\gamma\text{S}\) compared with ATP\(\gamma\text{S}\) suggested a specificity for guanine over adenosine nucleotides. When the dialyzed cytosol was used, the nucleoside triphosphate requirement was radically altered in favor of guanine nucleotides, and the effect was at the level of the \( V_{\text{max}} \). As with the dialyzed cytosol, the NTP specificity was independent of whether SDS alone or SDS plus diacylglycerol was used to activate.

The present studies indicate that the nucleoside triphosphate specificity is primarily for the guanine nucleotide, and imply that the effects of adenosine triphosphates in the undialyzed system were due to a NDP kinase activity which can transfer the \( \gamma \)-phosphoryl or \( \gamma \)-(thio)phosphoryl group from ATP or ATP\(\gamma\text{S}\) to GDP to form the corresponding GTP derivative. In dialyzed cytosol, free GDP is removed, thus eliminating this pathway. In support of this explanation, we demonstrated directly a NDP kinase activity in both cytosol and plasma membrane which was capable of transferring the thiophosphoryl group from ATP\(\gamma\text{S}\) to GDP. In addition, GDP restored the ability of ATP\(\gamma\text{S}\) to activate maximally. Seifert et al. (29) previously demonstrated a nucleoside diphosphate kinase activity in differentiated HL-60 cells. This group observed ATP\(\gamma\text{S}\) stimulation of superoxide generation in the arachidonate-activated cell-free system, and proposed that this was due to NDP kinase-catalyzed generation of GTP\(\gamma\text{S}\), based on a lack of activation by App(NH)p and inhibition by UDP. As in the Seifert studies, we also found UDP to be an effective inhibitor of ATP and ATP\(\gamma\text{S}\) stimulation of oxidase activity. However, stimulation by GDP was not observed in (29), perhaps because of endogenous GDP in undialyzed cytosols. In our experiments, we found a narrow window of concentrations in which GDP affected ATP\(\gamma\text{S}\) stimulation.
The reason for the narrow activity window for GDP is not clear, but may relate to inhibition of activity by excess GDP which may not be completely converted to GDP. We suggest that the presence of NDP kinase and variable endogenous nucleotide levels, which may occur using different preparation methods and dilutions, accounts for contradictory reports regarding the effects of ATP and ATPγS on activation.

Two quantitatively small but apparently anomalous results require explanation. First, in the dialyzed system with SDS plus diacylglycerol, there is a small residual stimulation by ATPγS, and to a lesser extent by ATP (Fig. 4, upper right panel). The stimulation retains the same EC50 for ATPγS, but shows a markedly lower Vmax compared with the undialyzed system. This observation could indicate a modest activating effect of a kinase. Alternatively, there may be residual GDP which can serve as phosphoryl acceptor. Although residual free GDP is not expected given the extensive dialysis protocol, a small quantity of tightly protein-bound GDP may be retained. In this regard, many G proteins are known to retain bound GDP. Although the classical mechanism for activation of G proteins involves dissociation of GDP with subsequent binding of GTP, recent evidence indicates that G can be physically associated with NDP kinase (48, 49) and that the latter can catalyze directly the phosphorylation of GDP by ATP or ATPγS without nucleotide exchange (50, 51). Thus, if residual GDP is bound to a G protein, ATP (or ATPγS) might serve as direct phosphoryl donor to the bound nucleotide. This mechanism could also account for a second apparent anomaly: while there is a marked specificity for GTPγS over ATPγS, there is a preference for ATP over GTP when native nucleotides are used (e.g. see Fig. 1). This could be explained if a G protein-associated NDP kinase shows a preference for ATP over GTP where native nucleotides are used (e.g. see Ref. 57). It will therefore be of interest to investigate the effects of diacylglycerol on the translocation of cytosolic components of the respiratory burst oxidase.

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