NADH oxidase activity of rat liver plasma membrane activated by guanine nucleotides

D. James MORRÉ,*‡ Michelle DAVIDSON,* Christoph GEILEN,* James LAWRENCE,* Gregory FLESHER,*† Ruth CROWE† and Frederick L. CRANE†

* Department of Medicinal Chemistry and † Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, U.S.A.

The activity of a hormone- and growth-factor-stimulated NADH oxidase of the rat liver plasma membrane responds to guanine nucleotides, but in a manner that differs from that of the classic trimeric and low-molecular-mass monomeric G-proteins. In the absence of added bivalent ions, both GTP and GDP as well as guanosine 5′-[γ-thio]triphosphate (GTP[γ-S]) but not guanosine 5′-[β-thio]diphosphate (GDP[β-S]) stimulate the activity over the range 1 μM to 100 μM. Other di- and tri-nucleotides also stimulate, but only at concentrations of 100 μM or higher. Added bivalent ions are not required either for NADH oxidation or guanine nucleotide stimulation. Bivalent ions ($Mg^{2+} > Mn^{2+} \geqslant Ca^{2+}$) alone stimulate only slightly at low concentrations and then inhibit at high concentrations. The inhibitions are augmented by GDP or GTP[γ-S] but not by GTP. Although the activity is the same, or less, in the presence of 0.5 mM $MgCl_2$, GTP at 1–100 nM and other nucleotides at 0.1 mM or 1 mM still stimulate in its presence. The NADH oxidase is activated by mastoparan but aluminum fluoride is weakly inhibitory. Cholera and pertussis toxins elicit only marginal responses. Both the Mg^{2+} and the GDP and GTP[γ-S] inhibitions (but not the GTP stimulations) shift to higher concentrations when the membrane preparations are first solubilized with Triton X-100. The results suggest a role for guanine nucleotides in the regulation of plasma membrane NADH oxidase, but with properties that differ from those of either trimeric or the low-molecular-mass G proteins thus far described.

INTRODUCTION

Guanine nucleotide-binding proteins (G-proteins) encompass several families of signal-coupling proteins that play key roles in many hormonal and sensory transduction processes in eukaryotes [1–3]. The trimeric G-proteins have a common oligomeric structure consisting of α, β and γ subunits that carry signals from membrane-bound receptors to effectors such as enzymes and ion channels [2]. The α subunits have a high-affinity binding site specific for GTP (or GDP) and a site for NAD-dependent ADP-ribosylation which is catalysed by bacterial toxins such as pertussis and/or cholera toxins. A large number of monomeric GTP-binding proteins have been described as well [3]. The latter group most often have molecular masses in the range of 20–30 kDa and include the *ras* superfamily of proto-oncogenes. A common feature of all GTP-binding proteins is that they undergo two alternate conformations depending on the ligand. The GTP-bound form is active, whereas after hydrolysis of GTP to GDP, the resultant GDP-bound form is inactive. The switch between these two states appears as the critical element in the ability of these proteins to control cellular functions.

Our laboratory has described recently a hormone- and growth-factor-responsive NADH oxidase activity of the plasma membrane of rat liver [4–6]. To characterize further this activity, we have investigated the response of the activity to guanine nucleotides, bivalent ions and other effectors normally associated with regulation of the activity of G-proteins. The NADH oxidase appears to respond to guanine nucleotides and some, but not other, G-protein effectors. However, the response and its characteristics do not parallel exactly those of either the classic monomeric or trimeric GTP-binding proteins described previously.

MATERIALS AND METHODS

Purification of rat liver plasma membrane

The 5000 *g* pellet from the preparation of the Golgi apparatus [7] was the starting material. The fluffy layer which contains the Golgi apparatus fraction was mixed and withdrawn with a 1-mm-diam. pipette, and was excluded from the plasma membrane preparations. Cold 1 mM $NaHCO_3$ (5 ml) was added to each tube and the friable yellow-brown upper part of the pellet was resuspended with a penbrush, leaving the reddish tightly packed bottom part of the pellet undisturbed. The resuspended material was transferred to a centrifuge tube, and a second 5 ml of cold $NaHCO_3$ was added to collect the remaining friable material. The combined resuspended material was homogenized in aliquots of 5 ml each in a 30 ml stainless steel (Duragrind) homogenizer 20 times by hand. The homogenates were combined, diluted with cold 1 mM $NaHCO_3$ (1:1 dilution), and centrifuged at 6000 *g* in a HB 4 rotor for 15 min. The supernatant was discarded and the pellet was used for the two-phase separation.

The two-phase system contained 6.4% (w/w) Dextran T-500 (Pharmacia), 6.4% (w/w) poly(ethylene glycol) 3350 (Fisher), and 5 mM potassium phosphate buffer (pH 7.2) [8]. The homogenate (1 g) was added to the two-phase system and the weight of the system was brought to 8 g with distilled water. The tubes were inverted vigorously 40 times at 4 °C. The phases were separated by centrifugation at 750 rev./min (150 *g*) in a Sorvall HB rotor for 5 min. The upper phases were carefully withdrawn with a Pasteur pipette, divided in half and transferred into 40 ml plastic centrifuge tubes and diluted with cold 1 mM $NaHCO_3$ by filling the tubes. The plasma membrane was collected by centrifugation at 10000 *g* in a HB rotor for 30 min. Proteins were

Abbreviations used: GTP[γ-S], guanosine 5′-[γ-thio]triphosphate; GDP[β-S], guanosine 5′-[β-thio]diphosphate; NDP kinase, nucleoside diphosphate kinase.

‡ To whom correspondence should be addressed.

determined using the bicinchoninic acid (BCA) assay [9] with BSA as standard.

The plasma membrane preparations utilized in this study have been characterized extensively, based on both morphological and enzymic criteria [8,10]. From morphometric analysis using electron microscopy, the preparations contain 90±4% plasma membrane. Contaminants include mitochondria (4%), endoplasmic reticulum (3%) and trace amounts of nuclear envelope, Golgi apparatus, lysosomes, peroxisomes and unidentified membranes (combined total of 3%). Based on analyses of marker enzymes, the contamination by endoplasmic reticulum was estimated to be 3%, that of mitochondria 15% and that of Golgi apparatus 1%. The yield of plasma membranes was estimated to average 18%, based on recovery of enzyme markers.

NADH oxidase activity

The assay for the plasma membrane NADH oxidase was performed in 40 mM Tris/Mes buffer (pH 7.0) and 150 μM NADH in the presence of 1 mM KCN, the latter to inhibit any mitochondrial NADH oxidases contaminating the plasma membranes. Rotenone, 1–2 μM, has been used in place of KCN with comparable results. The assay was started by the addition of 0.1 mg of plasma membrane protein. Incubations were for 10 min with the reported rates being measured between 5 and 10 min after additions of nucleotide or other compounds (see below). The assay temperature was 37 °C. The reaction was monitored by the decrease in the absorbance at 340 nm with 430 nm as reference, using an SLM DW-2000 spectrophotometer in the dual-wavelength mode of operation, or at 340 nm using a Hitachi model U3210. The change of absorbance was recorded as a function of time using a chart recorder. The specific activity of the plasma membrane was calculated using an absorption coefficient of 6.21 $mM^{-1} \cdot cm^{-1}$ and expressed as nmol/min per mg of protein.

All oxidase rates reported are at steady state following a 10 min temperature equilibration of the system. All additions were monitored over at least 10 min of post-equilibration incubation. Values were averaged over the second 5 min. Results are averages of two (Figures 2, 3 and 6) or three experiments (Tables 1–4, Figures 1, 4 and 5) with two or three determinations in each experiment±mean average ($n = 2$) or S.D.s ($n = 3$) among experiments.

Assay of adenylate cyclase activity

To assay adenylate cyclase activity in the isolated rat liver plasma membranes, an isotopic dilution method was used. Approx. 500 μg of plasma membrane was incubated in the presence of 2 mM $MgSO_4$, 2 mM ATP, 10 mM theophylline, 1 mM EDTA and 0.1 M Tris, pH 7.5. Also included was an ATP regeneration system consisting of phosphoenolpyruvate (2.5 mM) and pyruvate kinase (15 units/ml). Components were combined in the ratio of 50 μl of plasma membrane preparation and 50 μl of buffer containing the above-mentioned ATP regeneration system. The final volume was 100 μl and was incubated for 20 min at 30 °C. The reaction was stopped by placing the tubes in a boiling water-bath for 3 min. Flocculated membranes were removed by centrifugation and the supernatants were kept for cyclic AMP determination. Cyclic AMP was quantified using a commercially obtained assay system based on a cyclic AMP-binding protein from bovine muscle and [8-^{3}H]adenosine 3′,5′-cyclic phosphate (Amersham TRK 432) according to the manufacturer's instructions.

Toxin studies

Cholera toxin (0.05 μg/ml) was activated by incubation for 30 min at 30°C in the presence of 25 mM Tris/Mes and 25 mM dithiothreitol, pH 7 [11]. For ADP ribosylation, a mixture of 5 μl of purified recombinant ARF (ADP ribosylation factor) (0.03 μg/μl, a gift from Dr. Joel Moss, NHLBI, NIH, Bethesda, MD, U.S.A.), 20 μl of NAD^+ (60 μM), 20 μl of plasma membrane (5 μg/μl), 20 μl of activated toxin and 60 μl of cholera toxin buffer were incubated on ice for 3 h [11,12]. At the end of the incubation period NADH oxidase activity was measured. The toxin buffer contained 600 mM potassium phosphate, 20 mM $MgCl_2$, 20 mM thymidine, 2 mM ATP and 0.2 mM GTP.

Pertussis toxin (Sigma, St. Louis, MO, U.S.A.) was activated at a concentration of 0.05 μg/μl using the same buffer as used for cholera toxin activation [11]. For ADP ribosylation, the incubations contained 10 μM NAD^+, 60–120 μg of plasma membrane, 10 mM thymidine, 1.0 mM ATP, 1.0 mM EDTA and 0.1 mM GTP. The mixture was incubated on ice with shaking for 3 h. At the end of the incubation period, the mixture was assayed for NADH oxidase activity. Control incubations were made with membrane alone, or membranes incubated with toxin buffer but without toxin added.

RESULTS

Stimulation by guanine nucleotides

The NADH oxidase of rat liver was stimulated by guanine nucleotides, both GTP and GDP, over a wide range of

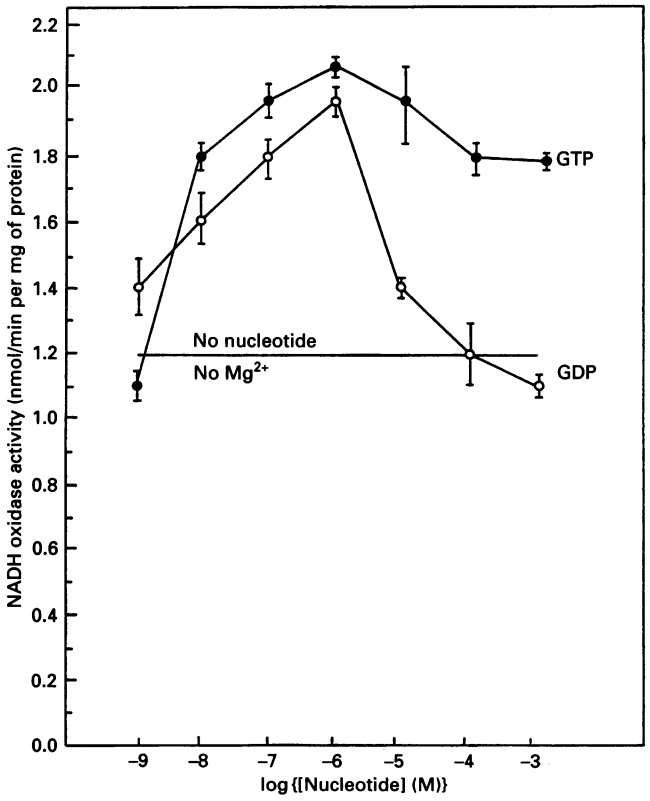

Figure 1 Steady-state NADH oxidase activities of intact rat liver plasma membranes respond to guanine nucleotides in the absence of added bivalent ions

The activity in the absence of added nucleotide was 1.2±0.1 nmol/min per mg of protein.

Table 1 Stimulation of rat liver plasma membrane NADH oxidase by 10 μM biferric transferrin (BFTF) in the presence or absence of 1 μM or 10 μM GTP or 10 μM GDP

Addition	NADH oxidase activity (nmol/min per mg of protein)
None	1.2±0.5
+10 μM BFTF	1.85±0.2
+1 μM GTP	2.0±0.2
+10 μM GTP	2.3±0.4
+1 μM BFTF+1 μM GTP	2.5±0.3
+1 μM BFTF+10 μM GTP	3.1±0.3
+10 μM GDP	1.3±0.15
+1 μM BFTF+10 μM GDP	1.9±0.15

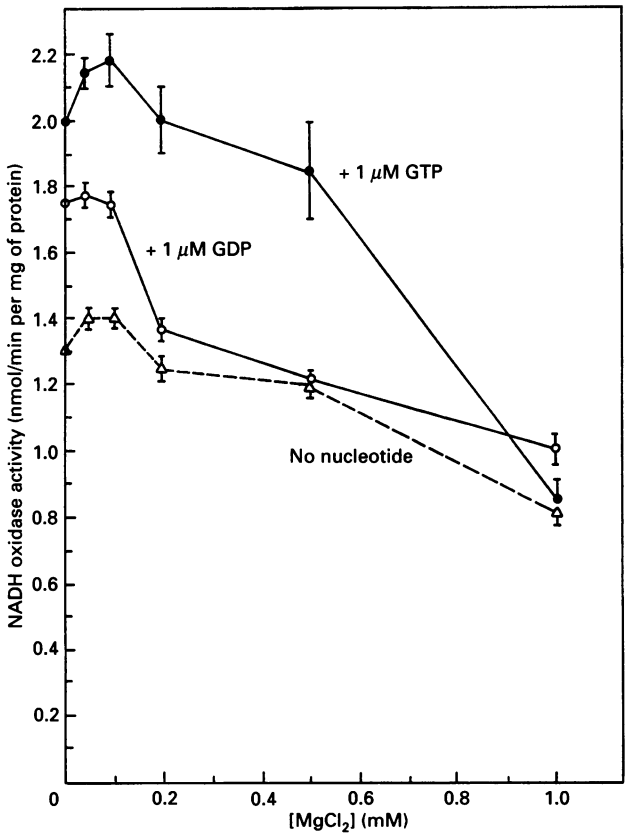

Figure 2 Response of NADH oxidase activities after 5 min of preincubation in the presence of various concentrations of $MgCl_2$, comparing absence of nucleotide with either 1 μM GDP or GTP

concentrations in the absence of added Mg^{2+} (Figure 1). The optimum for both nucleotides was about 1 μM. At concentrations greater than 1 μM, the stimulatory effect of GDP was lost, but that for GTP was retained (Figure 1).

The stimulation of plasma membrane NADH oxidase was not dependent upon the presence of growth factors. GTP stimulated in both the presence or absence of biferric transferrin (Table 1) and the stimulations by biferric transferrin and GTP were approximately additive.

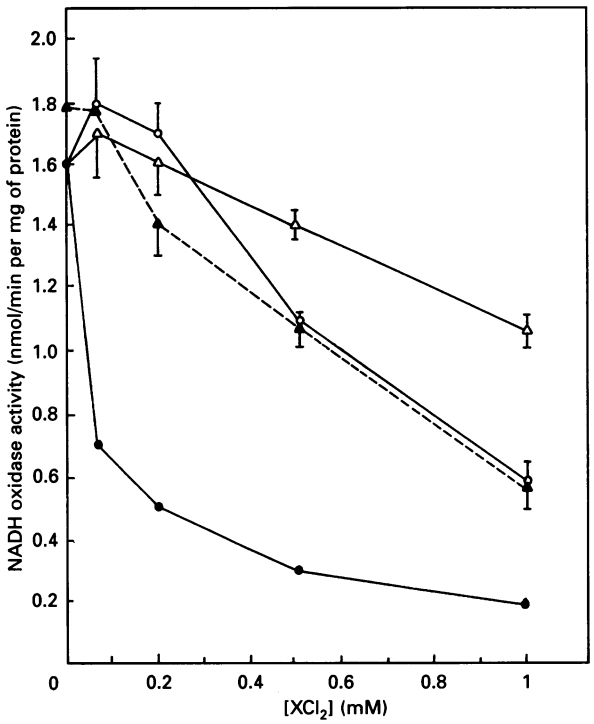

Figure 3 NADH oxidase inhibition by preincubation with bivalent cations at 0.05 mM for 50 min followed by 10 min incubations at the ion concentrations shown

For the broken curve, the plasma membranes were solubilized with 0.1% Triton X-100 to evaluate the requirement for an intact membrane for the ion response. Key to symbols: △, $CaCl_2$; ○, $MnCl_2$; ▲, $MgCl_2$+0.1% Triton X-100; ●, $MgCl_2$.

Response to bivalent ions

Mg^{2+} added to the membranes stimulated activity slightly at concentrations of 0.1 mM or less, but at concentrations of 0.2 mM or higher it negated the stimulatory effect of GDP, but not that of GTP (Figure 2). The inhibition by Mg^{2+} appeared to be time-dependent and even 0.05 mM Mg^{2+} became inhibitory with prolonged preincubation (Figure 3).

The inhibition of the NADH oxidase was shifted to higher concentrations of bivalent ions if the membranes were first solubilized with 0.1% Triton X-100 (Figure 3). Concentrations of $MgCl_2$ of 0.2 mM or higher were inhibitory, however, even with the solubilized membranes (Figure 3). With solubilized membranes, $MgCl_2$, $MnCl_2$ and $CaCl_2$ all inhibited in a similar manner with comparable dose dependencies (results not shown).

The stimulations of NADH oxidase by 1 μM or less of GTP or GDP were specific for these nucleotides. GMP stimulated the activity by 50% at 10 μM. Guanosine and guanine were without effect in the absence of Mg^{2+} and, in the presence of 0.05 mM $MgCl_2$, stimulated at high concentrations.

Effects of other nucleotides

NADH oxidase was stimulated by all di- and tri-nucleotides tested at concentrations of 0.1 mM and 1 mM (Figure 4), by CDP and CTP at 10 μM (Figure 4), but only by GTP and GDP at 1 μM (Figure 1). The pattern of response to nucleotides other than guanine nucleotides was similar in either the presence or absence of 0.05 mM $MgCl_2$ and, except for UTP and UDP, activities were decreased when the nucleotides were tested in the

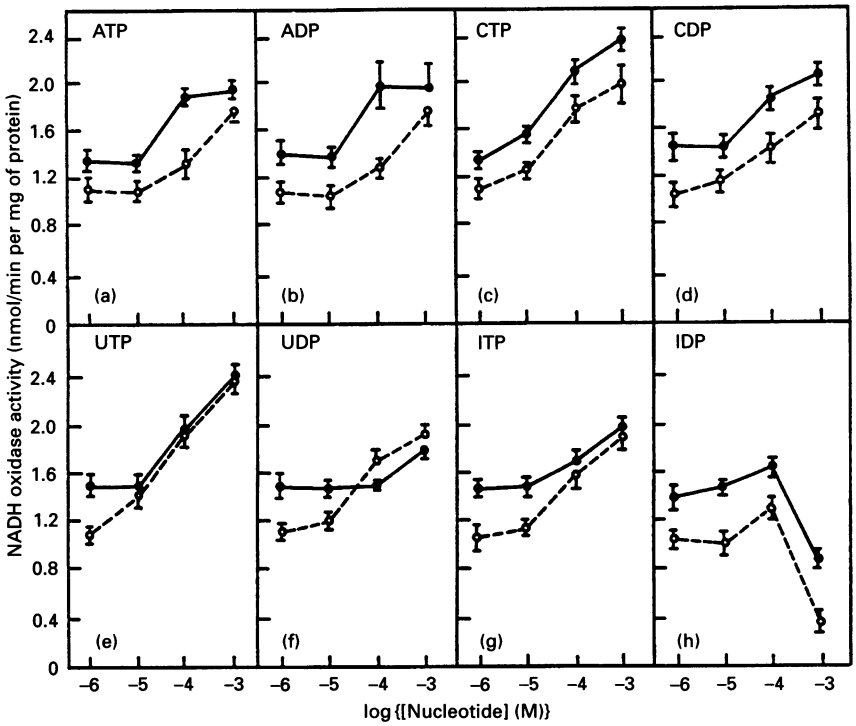

Figure 4 Response of NADH oxidase activity to nucleotide di- and tri-phosphates in the absence (solid curves and closed symbols) and presence (broken lines and open symbols) of 0.05 mM $MgCl_2$

The specific activity in the absence of nucleotide and added $MgCl_2$ was 1.6 ± 0.1 nmol/min per mg of protein. The specific activity in the absence of nucleotide but in the presence of added 0.05 mM $MgCl_2$ was 1.3 ± 0.15 nmol/min per mg of protein. Measurements were initiated after a 15 min preincubation with or without $MgCl_2$.

Table 2 Response of adenylate cyclase activity of isolated rat liver plasma membranes to 3 μM glucagon in the presence of 20 μM GTP, GTP[γ-S], GDP or GDP[β-S] as well as $[AlF_4]^-$ (50 μM $Al^+ + 20$ mM F^-) or 10 μM mastoparan alone

Addition	Adenylate cyclase activity (pmol of cyclic AMP/mg of protein)
None	2.3 ± 0.7
Glucagon	2.8 ± 0.9
Glucagon + GTP	5.0 ± 0.9
Glucagon + GTP[γ-S]	5.4 ± 1.0
Glucagon + GDP	6.0 ± 0.5
Glucagon + GDP[β-S]	2.0 ± 0.4
$[AlF_4]^-$	6.1 ± 9.6
Mastoparan	5.8 ± 1.7

Table 3 Response of NADH oxidase of rat liver plasma membranes to GTP and GDP[β-S] alone and in combination

Addition	NADH oxidase activity (nmol/min per mg of protein)
None	1.2 ± 0.2
+ GDP[β-S] (10 μM)	1.1 ± 0.2
+ GTP (10 μM)	1.3 ± 0.15
+ GTP (10 μM) + GDP[β-S] (10 μM)	1.1 ± 0.1
+ GTP (100 μM)	1.6 ± 0.1
+ GTP (100 μM) + GDP[β-S] (10 mM)	1.2 ± 0.1

presence of 0.05 mM $MgCl_2$ (Figure 4). When tested with Triton-solubilized membranes, UTP and CTP were without effect below 1 μM, were slightly stimulatory at 1 μM, and inhibitited the activity by about 50% at concentrations of 10 μM to 1 mM (results not shown). The other nucleotides were not investigated with Triton-solubilized membranes.

Comparisons to adenylate cyclase and response to guanosine 5′-[β-thio]diphosphate (GDP[β-S])

Adenylate cyclase activity of the isolated rat liver plasma membranes was stimulated by added glucagon in the presence of GTP as well as guanosine 5′-[γ-thio]triphosphate (GTP[γ-S]) (Table 2). GDP also stimulated activity but the non-metabolizable GDP analogue GDP[β-S] did not. Similarly GDP[β-S] did not stimulate NADH oxidase and when pre-incubated with plasma membranes, GDP[β-S] prevented GTP stimulation (Table 3). These findings taken together suggest that the GDP stimulations of NADH oxidase and of adenylate cyclase observed may have resulted from conversion of GDP into GTP via the enzyme nucleoside diphosphate kinase (see the Discussion).

GDP inhibition and response to GTP[γ-S]

The inhibitions of NADH oxidase by GDP but not by GTP were augmented in the presence of 0.05 mM $MgCl_2$ (Figure 5). GTP

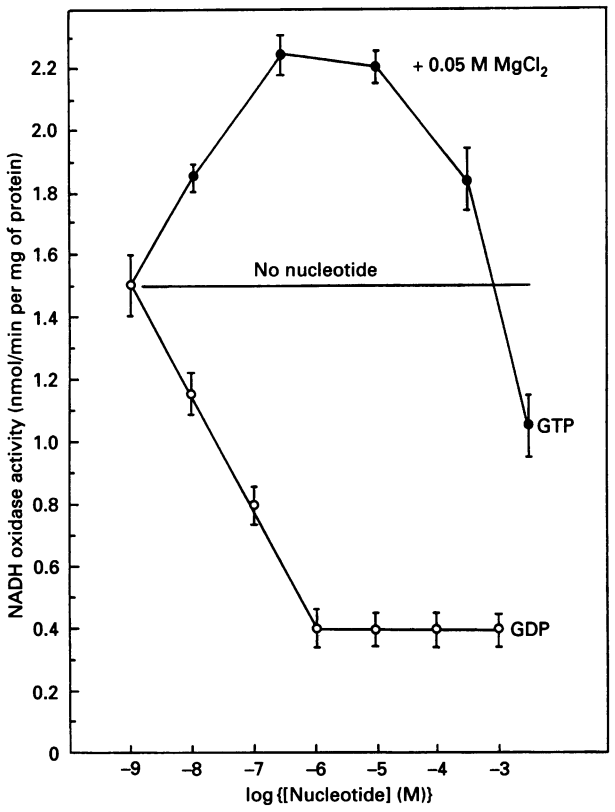

Figure 5 Concentration dependence of the response of NADH oxidase activities to GTP (stimulation) and GDP (inhibition) in the presence of 0.05 mM $MgCl_2$

Compare with Figure 1 in the absence of added $MgCl_2$, where the activity was stimulated by both GTP and GDP. The specific activity in the absence of added nucleotide was 1.5 ± 0.1 nmol/min per mg of protein.

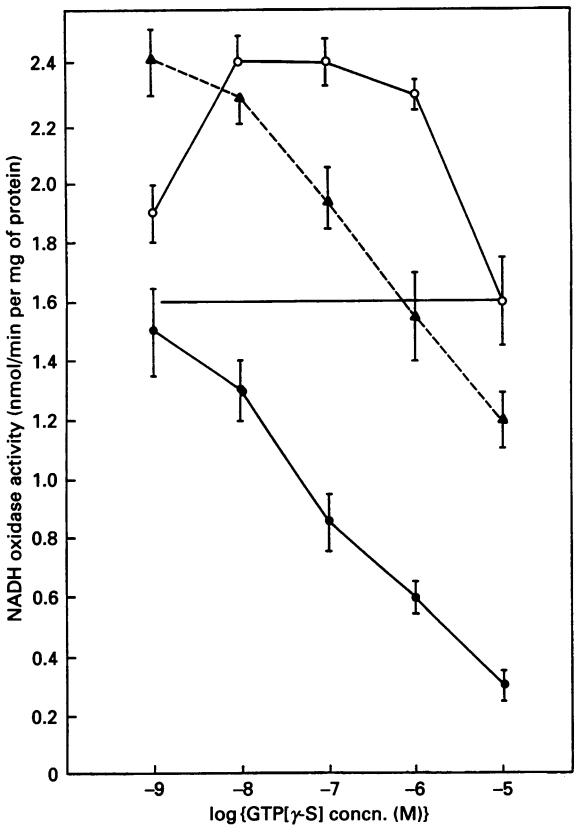

Figure 6 NADH oxidase of rat liver plasma membranes and response to GTP[γ-S] in the presence (●) and absence (○) of 0.05 mM $MgCl_2$

The broken curve illustrates the response to GTP[γ-S] in the presence of 0.05 mM $MgCl_2$ after the plasma membranes were solubilized with 0.1% Triton X-100. The specific activity in the absence of added nucleotide was 1.6 ± 0.15 nmol/min per mg of protein. Key to symbols: –○–, GTP[γ-S] in absence of 0.05 mM $MgCl_2$; –●–, GTP[γ-S] in presence of 0.05 mM $MgCl_2$; --▲--, GTP[γ-S] + 0.05 mM $MgCl_2$ after solubilization with Triton X-100.

Table 4 Stimulation of NADH oxidase of rat liver plasma membranes by mastoparan (*n*, number of experiments)

Mastoparan (μM)	*n*	NADH oxidase activity (nmol/min per mg of protein)
0	5	1.35 ± 0.12
1	3	1.52 ± 0.36
5	4	2.23 ± 0.57
10	3	1.52 ± 0.55

stimulations were still observed with Mg^{2+} present but with GDP the result was inhibition, with 50% inhibition at about 0.1 μM GDP (Figure 5).

An interaction of guanine nucleotide with $MgCl_2$ was seen also with GTP[γ-S] (Figure 6). In the presence of 0.05 mM added Mg^{2+}, the NADH oxidase activity of the rat liver plasma membranes was inhibited in proportion to the logarithm of GTP[γ-s] concentration between 0.001 mM and 10 mM. In the absence of $MgCl_2$, GTP[γ-S] stimulated the NADH oxidase activity. As with the effect of $MgCl_2$ on the basal oxidase activity, the inhibitions in the presence of GTP[γ-S] were reduced by Triton X-100 treatment. A 1000-fold higher concentration of GTP[γ-S] was required in the presence of 0.05 mM $MgCl_2$ to inhibit NADH oxidase when the plasma membranes were first solubilized in 0.1% Triton X-100 (Figure 6).

Response to toxins, mastoparan and aluminium fluoride

NADH oxidase was only marginally sensitive to preincubation with either pertussis or cholera toxin. Preincubation with cholera toxin resulted in a small inhibition (20%) that occurred progressively over 50 min of incubation with NADH compared with toxin buffer alone. Pertussis toxin inhibited by 15% compared with toxin buffer alone. Mastoparan, an ampipathic peptide that mimics G-protein receptors, stimulated the NADH oxidase of rat liver plasma membrane 1.65-fold at 5 μM (Table 4). Aluminium fluoride was largely without effect or was inhibitory. At concentrations up to 40 μM Al^{3+} + 6 mM F^- ($[AlF_4]^-$) no responses were observed. Above 40 μM Al^{3+}, $[AlF_4]^-$ was inhibitory, reaching an inhibition of about 40% at 0.4 mM Al^{3+} (results not shown).

DISCUSSION

The discovery by Rodbell and colleagues [13] that GTP was essential to promote agonist stimulation of adenylate cyclase in membranes led to the identification of G-proteins as key

participants in hormone action. G-proteins mediate the effect of agonists, working through their specific receptors, on the activity of effector molecules, which may be enzymes or ion channels. One class of G-proteins, the trimeric G-proteins, is distinguished by α polypeptides that bind and hydrolyse GTP together with β and γ peptides that anchor the complex to the membrane. Trimeric G-proteins share other unique or unusual characteristics. For example, they are activated by fluoride plus aluminium. Also distinctive is that the α subunits of individual G-proteins are substrates for ADP-ribosylation catalysed by bacterial toxins.

Among the criteria for involvement of a G-protein is that both an appropriate ligand for the receptor of interest and GTP are required to initiate the response in question. The response can be provoked independently of receptors by inclusion of non-hydrolysable analogues of GTP (GTP[γ-S]) or $[AlF_4]^-$. Mastoparan, a cationic, amphophilic tetradecapeptide, also stimulates guanine nucleotide exchange by G-proteins, in a manner similar to that of G-protein-coupled receptors [14,15]. Mastoparan has the ability to interact not only with trimeric G-proteins but also with low-molecular-mass G-proteins [16]. This report describes a response of a hormone- and growth-factor-responsive NADH oxidase of the rat liver plasma membrane that distinguishes it from the classic trimeric G-proteins. It is stimulated by both GTP and mastoparan but not by $[AlF_4]^-$ in the absence of growth factor.

In the absence of added Mg^{2+} ions, the NADH oxidase of rat liver plasma membranes was stimulated by low concentrations (1–100 nM) of GTP and GDP, as well as by GTP[γ-S]. Other nucleotides also were stimulatory but required millimolar concentrations for comparable activity increases. These stimulations were retained by the NADH oxidase of detergent-solubilized membranes.

In the presence of relatively low concentrations of added Mg^{2+} (0.05 mM), the NADH oxidase activity exhibited a transient stimulation followed by a time-dependent inhibition. This inhibition was augmented by both GDP and GTP[γ-S], with 50% inhibition in the range of 0.1 μM. Tight binding of both GDP and GTP[γ-S] by both low-molecular-mass monomeric and trimeric G-proteins required Mg^{2+} [2].

The Mg^{2+}-dependent inhibitions of the plasma membrane NADH oxidase were largely abolished if the membranes were first solubilized with detergent. In contrast, the GTP-stimulation was reduced but not abolished either in the presence of Mg^{2+} or in detergent-solubilized preparations. These findings suggest that the pronounced GDP + Mg^{2+} or GTP[γ-S] + Mg^{2+} inhibitions, but not the GTP stimulations of the oxidase, require some conformational constraints provided by the intact membrane. These might be either with the NADH oxidase itself or between the NADH oxidase and another interacting component that binds GDP and GTP[γ-S] with high affinity. When in the GDP-bound form or where turnover is blocked by GTP[γ-S], the activity of the NADH oxidase is blocked also. However, once the membranes are detergent-solubilized, the associations are no longer maintained and GDP plus Mg^{2+} or GTP[γ-S] plus Mg^{2+} no longer inhibit. We are unable to determine if this alteration in response results from a failure of the NADH oxidase to bind GDP or GTP[γ-S] in the presence of Mg^{2+} following the detergent treatment. Alternatively, the detergent treatment might result in the disruption of a functional association between the NADH oxidase and a regulatory G-protein or non-catalytic subunit of the intact membrane required for inhibition. There may be sufficient bivalent ions, on the other hand, bound to the intact membrane to support GTP binding, even in the absence of added ions.

The stimulation of NADH oxidase by GDP is somewhat problematic but is probably due to the presence in rat liver plasma membranes of nucleoside diphosphate kinase (NDP kinase). This is supported by the observations that adenylate cyclase activity activated by glucagon was stimulated equally by GDP and GTP in the isolated rat liver plasma membranes, whereas GDP[β-S], which cannot be phosphorylated by NDP kinase, did not stimulate adenylate cyclase.

NDP kinase is a ubiquitous enzyme that catalyses the transfer of the γ-phosphate of 5′-triphosphate nucleotides to 5′-diphosphate nucleotides via a high-energy phosphorylated enzyme intermediate [17]. Others have reported that GDP was as effective as GTP in hormone-dependent activation of adenylate cyclase even in the absence of an ATP-regenerating system [13,18,19]. These findings have been interpreted to indicate that the GDP effect results from the GDP conversion of the added GDP into GTP through the action of NDP kinase. Kimura and Shimada [20] reported that NDP kinase activity was associated with plasma membranes in addition to the cell cytosol.

The NADH oxidase activity was stimulated by mastoparan, but was affected only slightly by either pertussis or cholera toxins. There were no simulations by $[AlF_4]^-$, which in the presence of GDP is known to activate both inhibitory and stimulatory trimeric G-proteins by mimicking the γ-phosphate moiety of GTP [21]. $[AlF_4]^-$ does not affect the low-molecular-mass *ras*-like GTPases.

We also used bacterial toxins which specifically catalyse the ADP-ribosylation of different α subunits of trimeric G-proteins [1]. Thus cholera toxin catalyses the ADP-ribosylation of stimulatory α subunits ($G\alpha_s$) [22], whereas pertussis toxin catalyses the ADP-ribosylation of different α subunits, including inhibitory ($G\alpha_i$) and other ($G\alpha_0$) subunits [23,24]. The NADH oxidase activity of intact rat liver membranes was little affected by either toxin.

Taken together the findings suggest that the activity measured as NADH oxidase may represent a unique guanine nucleotide-modulated protein of the mammalian plasma membrane. The activity is stimulated both by low concentrations of GTP and mastoparan, but is distinguished from timeric G-proteins in its failure to be stimulated by $[AlF_4]^-$, or to be inhibited by bacterial toxins. The inhibition by GTP[γ-S] and GDP in the presence of magnesium, while characteristic of monomeric G-proteins, required the presence of an intact membrane and was not exhibited by detergent-solubilized NADH oxidase, which provides evidence for potential coupling of NADH oxidase, as well, to more conventional signal-coupling G-proteins of the membrane.

The microbicidal activity of phagocytic white blood cells has long been related to a unique capacity for superoxide production in response to a number of stimuli [25,26]. Activation of this process, known as the respiratory burst, involves the assembly of an NADPH oxidase from several membrane-bound and cytosolic components [27,28]. Among these is a small G-protein [29].

In addition to having different reduced pyridine nucleotide substrates, the liver NADH oxidase differs in several important respects from the neutrophil NADPH oxidase. The liver NADH oxidase catalyses the bivalent reduction of molecular oxygen to water [30] whereas the neutrophil NADH oxidase catalysed a univalent reduction of molecular oxygen to form a superoxide anion [26]. Activation of the liver NADH oxidase occurs independently of cytosolic components whereas activation of the neutrophil NADH oxidase involves at least three cytosolic components [29,31,32]. The liver NADH oxidase also appears to lack a comparable counterpart to the flavoprotein cytochrome b_{558} component of the complete neutrophil NADH oxidase [27].

The purified ADP-ribosylation factor was a gift from Dr. Joel Moss, NHLBI, National Institutes of Health, Bethesda, MD, U.S.A. Work supported in part by NIH GM 44675 and Lilly Research Laboratories, Indianapolis. C.G. was the recipient of a grant from the Deutsche Forschungsgemeinschaft. We thank Dr. Warren MacKellar, Dr. Andrew Brightman and Professor Dr. Werner Reutter for helpful discussions and support of specific aspects of the work.

REFERENCES

1 Kaziro, Y., Itoh, H., Kozasa, T., Nakafuku, M. and Satoh, T. (1991) Annu. Rev. Biochem. **60**, 349–400
2 Gilman, A. G. (1987) Annu. Rev. Biochem. **56**, 615–649
3 Bourne, H. R., Sanders, D. A. and McCormick, F. (1991) Nature (London) **349**, 117–127
4 Morré, D. J., Crane, F. L., Eriksson, L. C., Löw, H. and Morré, D. M. (1991) Biochim. Biophys. Acta **1057**, 140–146
5 Brightman, A. O., Wang, J., Miu, R.-K., Sun, I. L., Barr, R., Crane, F. L. and Morré, D. J. (1992) Biochim. Biophys. Acta **1105**, 109–117
6 Bruno, M., Brightman, A. O., Lawrence, J., Werderitsh, D., Morré, D. M. and Morré, D. J. (1992) Biochem. J. **284**, 625–628
7 Morré, D. J. (1971) Methods Enzymol. **22**, 130–148
8 Morré, D. J. and Morré, D. M. (1989) BioTechniques **7**, 946–958
9 Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, E. K., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985) Anal. Biochem. **100**, 76–85
10 Navas, P., Nowack, D. D. and Morré, D. J. (1989) Cancer Res. **49**, 2146–2147
11 Ribeiro-Neto, F. A. P., Mattera, R., Hildebrandt, J. D., Codina, J., Field, J. B., Birnbauer, L. and Sekura, R. D. (1985) Methods Enzymol. **109**, 566–572
12 Kahn, R. A. (1991) Methods Enzymol. **195**, 233–242
13 Rodbell, M., Birnbaumer, L., Phol. S. L. and Krans, H. M. J. (1971) J. Biol. Chem. **246**, 1877–1882
14 Higashijima, T., Uzi, S., Nakajima, T. and Ross, E. M. (1988) J. Biol. Chem. **263**, 6491–6494
15 Higashijima, T., Graziano, M. P., Suga, H., Kainosho, M. and Gilman, A. G. (1991) J. Biol. Chem. **266**, 3396–3401
16 Koch, G., Haberman, B., Mohr, C., Just, I. and Aktories, K. (1991) FEBS Lett. **291**, 336–340
17 Parks, R. E., Jr. and Agarwal, R. P. (1973) in The Enzymes (Boyer, P. D., ed.), vol. 8, pp. 307–333, Academic Press, New York
18 Kimura, N. and Nagata, N. (1977) J. Biol. Chem. **252**, 3829–3835
19 Kikkawa, S., Takahashi, K., Takahashi, K.-I., Shimada, N., Ui, M., Kimura, N. and Katada, T. (1990) J. Biol. Chem. **265**, 21536–21540
20 Kimura, N. and Shimada, N. (1990) Biochem. Biophys. Res. Commun. **168**, 99–106
21 Higashijima, T., Burnier, J. and Ross, E. M. (1990) J. Biol. Chem. **265**, 14176–14186
22 Cassel, D. and Pfeuffer, T. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 2669–2673
23 Hazeki, U. and Ui, M. (1981) J. Biol. Chem. **256**, 2856–2862
24 Sternweis, P. C. and Robinshaw, J. D. (1984) J. Biol. Chem. **259**, 13806–13813
25 Klebanoff, S. J. (1980) Ann. Intern. Med. **93**, 480–489
26 Babior, B. M. (1984) J. Clin. Invest. **73**, 599–601
27 Segal, A. W. (1989) J. Clin. Invest. **83**, 1785–1793
28 Smith, R. M. and Curnutte, J. T. (1990) Blood **77**, 673–686
29 Abo, A., Pick, E., Hall, A., Totty, N., Teahan, C. G. and Segal, A. W. (1991) Nature (London) **353**, 668–670
30 Morré, D. J. and Brightman, A. O. (1991) J. Bioenerg. Biomemb. **23**, 469–489
31 Nunoi, H., Rotrosen, D., Gallin, J. I. and Malech, H. L. (1988) Science **242**, 1298–1301
32 Volpp, B. D., Nauseef, W. M. and Clark, R. A. (1988) Science **242**, 1295

Received 21 August 1992/4 January 1993; accepted 13 January 1993