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NADH Oxidase Activity of Plasma Membranes of Soybean Hypocotyls Is Activated by Guanine Nucleotides

D. James Morré*, Andrew O. Brightman, Rita Barr, Michelle Davidson, and Frederick L. Crane

Department of Medicinal Chemistry (D.J.M., A.O.B., M.D.) and Department of Biological Sciences (R.B., F.L.C.), Purdue University, West Lafayette, Indiana 47907

The activity of an auxin-stimulated NADH oxidase of the plasma membrane of hypocotyls of etiolated soybean (*Glycine max* Merr.) seedlings responded to guanine and other nucleotides, but in a manner that differed from that of enzymes coupled to the classic trimeric and low molecular weight monomeric guanine nucleotide-binding proteins (G proteins). In the presence and absence of either auxin or divalent ions, both GTP and GDP as well as guanosine-5′-O-(3-thiotriphosphate) (GTP-γ-S) and other nucleoside di- and triphosphates stimulated the oxidase activity over the range 10 μM to 1 mM. GTP and GTP-γ-S stimulated the activity at 10 nM in the absence of added magnesium and at 1 nM in the presence of added magnesium ions. Other nucleotides stimulated at 100 nM and above. The NADH oxidase was stimulated by 10 μM mastoparan and by 40 μM aluminum fluoride. Neither cholera nor pertussis toxins, tested at a concentration sufficient to block mammalian G protein function, inhibited the activity. Guanosine 5′-O-(2-thiodiphosphate) (GDP-β-S) did not stimulate activity, suggesting that the stimulation in response to GDP may be mediated by a plasma membrane nucleoside diphosphate kinase through conversion of GDP to GTP. Auxin stimulation of the NADH oxidase was unaffected by nucleotides at either high or low nucleotide concentrations in the absence of added divalent ions. However, pretreatment of plasma membranes with auxin increased the apparent affinity for nucleotide binding. This increased affinity, however, appeared not to be the mechanism of auxin stimulation of the oxidase, since auxin stimulation was similar with or without low concentrations of guanine nucleotides. The stimulation by nucleotides was observed after incubating the membranes with 0.1% Triton X-100 prior to assay. The results suggest a role of guanine (and other) nucleotides in the regulation of plasma membrane NADH oxidase that differs from the interactions with G proteins commonly described for animal models.

G proteins encompass several families of signal-coupling proteins that play key roles in hormonal and sensory transduction processes in eukaryotes (Gilman, 1987; Bourne et al., 1991; Kaziro et al., 1991). 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 (Gilman, 1987). The α subunits have a high-affinity binding site for GTP (or GDP) and sites for NAD-dependent ADP-ribosylation, which is catalyzed by bacterial toxins such as pertussis toxin and/or cholera toxin. A large number of monomeric GTP-binding proteins have been described as well (Bourne et al., 1991). The latter group most often have molecular masses in the range of 20 to 30 kD and include products of the *ras* superfamily of proto-oncogenes. GTP-binding proteins of this molecular mass range have been observed in plant plasma membranes, based on GTP-γ-S binding (e.g. Blum et al., 1988; Zbell et al., 1990b). A common feature of all GTP-binding proteins is that they exist in 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. Mastoparan, a peptide toxin from wasp venom, constitutively activates G proteins by mimicking receptor-G protein interactions (Higashijima et al., 1988, 1990). Whereas the coupling of many regulatory responses of animal cells appears to include G protein involvement, comparable roles for G proteins in plants are only beginning to be elucidated.

Our studies have focused on the auxin-stimulated NADH oxidase of the plasma membrane of soybean hypocotyl (Morré et al., 1988; Morré and Brightman, 1991; Brightman et al., 1992). This activity has some of the characteristics of a G protein-coupled enzyme, but the response to guanine nucleotides differs from the usual mammalian paradigms in several important respects.

MATERIALS AND METHODS

Plant Materials

Soybean (*Glycine max* Merr.) seedlings were grown 4 to 5 d in darkness. Hypocotyl segments were excised under subdued light (1.5 $\mu E\ s^{-1}\ m^{-2}$) 5 mm below the cotyledons, and 2-cm segments were used for isolation of plasma membrane.

Plasma Membrane Isolation

Plasma membranes were isolated from an 8,000*g* to 50,000*g* pellet (microsomal membrane fraction) using aqueous polymer phase partition (Larsson et al., 1988). The system contained 6.4% (w/w) Dextran T-500 (Pharmacia), 6.4% (w/w) PEG (Fisher), 0.25 M Suc, and 5.0 mM potassium-phosphate buffer, pH 6.8. After mixing, the two phases were separated by centrifugation at 700*g* for 10 min. The plasma membrane vesicles partitioned to the upper phase, and the

* Corresponding author; fax 1–317–494–4007.

Abbreviations: BCA, bicinchoninic acid; G protein, guanine nucleotide-binding protein; GDP-β-S, guanosine 5′-O-(2-thiodiphosphate); GTP-γ-S, guanosine-5′-O-(3-thiotriphosphate); NDP, nucleoside diphosphate; PIP_2, phosphatidylinositol bisphosphate.

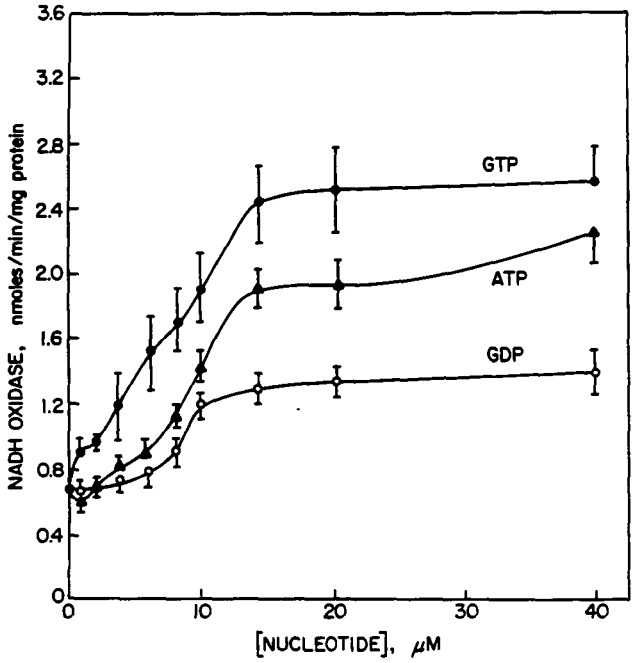

Figure 1. Nucleotide stimulation of NADH oxidase activity of isolated plasma membrane vesicles from etiolated hypocotyls of soybean.

other membranes remained in the lower phase. The upper-phase membranes were repartitioned twice against fresh lower phases and then diluted 8-fold with 0.25 M Suc, 25 mM Tris/Mes, pH 7.5, or water, and centrifuged for 30 min at 100,000*g* (Beckman SW-28). The plasma membrane pellets were suspended to 2 to 4 mg protein/mL and were used directly or stored frozen at −70°C. Unless indicated otherwise, for auxin experiments the membranes were refrozen at −20°C and thawed in a room-temperature water bath four times before use.

NADH Oxidase Assay

NADH oxidase activity was determined using a DW2000 spectrophotometer (SLM-Aminco) in the dual-wavelength mode as the decrease in A_{340} with A_{430} as reference. Rates were recorded with an attached HP plotter/printer. Reactions containing 20 mM Tris-Mes, pH 7, 150 μM NADH, and 1 mM KCN were at 25°C with constant stirring. Unless indicated otherwise, results are the average of two determinations from either three or two separate experiments ± SD ($n = 3$) or mean average ($n = 2$) deviation. Proteins were estimated by the BCA procedure (Smith et al., 1985). Plasma membrane (50–100 μg) was added to start the reactions. Mastoparan (Sigma) and its inactive analog, Mas-17 (Peninsula Laboratories), were dissolved in distilled water and stored at −80°C until use.

RESULTS

The NADH oxidase of plasma membranes of hypocotyls of etiolated soybean seedlings was stimulated by guanine nucleotides, both GTP and GDP, over the concentration range 1 to 40 μM in the absence of added Mg^{2+} or 2,4-D (Fig. 1). Other nucleotides such as ATP (Fig. 1) also stimulated over the same concentration range. The form of the concentration curves was complex, tending toward sigmoidal rather than hyperbolic or toward a series of sigmoidal curves suggestive of complex nucleotide-binding interactions. Of the nucleotides tested in the absence of magnesium, GTP not only exhibited both the greatest stimulation in the range 1 to 10 μM but stimulated at the lowest concentrations. ATP at 1 μM had a tendency to inhibit (Fig. 1).

NADH oxidase was not strongly affected by divalent ions (Fig. 2A). At a near optimal concentration of 0.01 mM, Mg^{2+} ions stimulated the NADH oxidase activity about 30%. The maximum degree of stimulation by GTP was similar in the presence and absence of Mg^{2+} (Fig. 2B). Results equivalent to those with Mg^{2+} were obtained with Ca^{2+} (Fig. 2A) and Mn^{2+} (not shown).

When compared in the presence and absence of 0.01 mM Mg^{2+} (Fig. 2B), the major difference in activity was seen at suboptimal GTP concentrations where activity normally was greater (15–45% with 10 nM GTP) in the presence of Mg^{2+} than in its absence. Similar results were found for GDP (Fig.

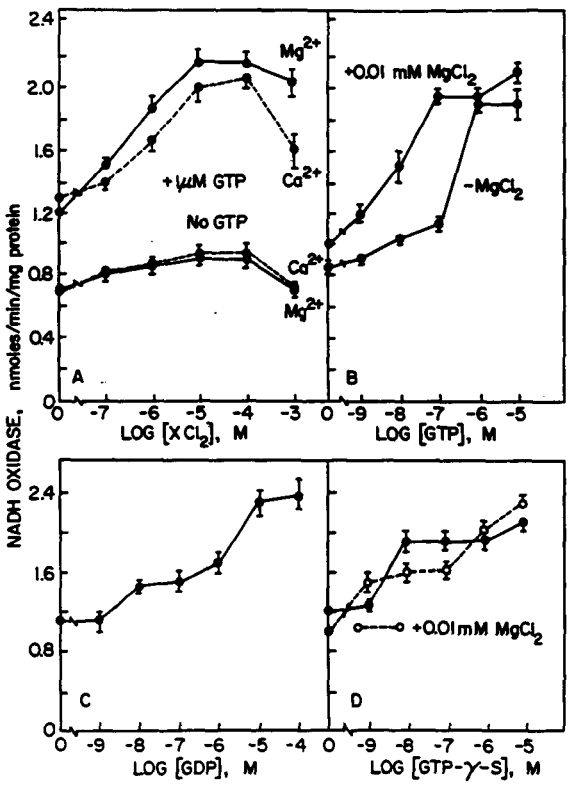

Figure 2. Effect of divalent ions and guanine nucleotides alone and in combination on NADH oxidase. A, Effect of divalent ions in the absence (lower curves) and presence (upper curves) of 1 μM GTP. B, GTP stimulation of NADH oxidase activity in the presence and absence of 0.01 mM $MgCl_2$. C, Response of NADH oxidase activity to GDP in the presence of 0.01 mM $MgCl_2$. D, Stimulation of NADH oxidase activity by the nonhydrolyzable analog of GTP, GTP-γ-S, in the presence (○) and absence (●) of 0.01 mM $MgCl_2$.

Table I. *Specific activities of NADH oxidase determined in the presence or absence of 10 nM nucleotides*

Nucleotide	Addition	No Nucleotide	10 nM Nucleotide	Ratio
GTP	None	0.75 ± 0.015	1.0 ± 0.04	1.3
	0.01 mM $MgCl_2$	0.8 ± 0.021	1.15 ± 0.02	1.4
	0.1% Triton X-100	0.6 ± 0.025	0.7 ± 0.02	1.2
GDP	None	0.7 ± 0.025	0.7 ± 0.03	1.0
	0.01 mM $MgCl_2$	0.6 ± 0.03	0.7 ± 0.05	1.2
	0.1% Triton X-100	0.65 ± 0.03	0.65 ± 0.02	1.0
ATP	None	0.65 ± 0.05	0.65 ± 0.05	1.0
	0.01 mM $MgCl_2$	0.6 ± 0.025	0.6 ± 0.025	1.0
	0.1% Triton X-100	0.7 ± 0.02	0.7 ± 0.04	1.0
ADP	None	0.6 ± 0.03	0.6 ± 0.03	1.0
	0.1% Triton X-100	0.7 ± 0.03	0.7 ± 0.04	1.0
GMP	None	0.7 ± 0.025	0.7 ± 0.02	1.0
Guanosine	None	0.5 ± 0.02	0.45 ± 0.02	0.9
Guanine	None	0.5 ± 0.025	0.55 ± 0.025	1.1

2C, Table I) and the nonhydrolyzable analog of GTP, GTP-γ-S (Fig. 2D). With all three nucleotides, a response was seen at 10 nM nucleotide or lower in the presence of Mg^{2+} (Table I, Fig. 2D). However, as summarized in Table I, only GTP stimulated the NADH oxidase at a concentration of 10 nM in the absence of Mg^{2+}. With $MgCl_2$ present, the stimulation by GTP was increased at 10 nM and stimulation was now observed as well at a concentration of 1 nM for GTP (Fig. 2B) and GTP-γ-S (Fig. 2B) but not at 1 nM with GDP (Fig. 2C) or other nucleotides (e.g. ATP, Table I).

Mastoparan, an ampipathic peptide that mimics G protein receptors, stimulated the NADH oxidase of soybean plasma membranes in a dose-dependent manner (Table II). The stimulation by mastoparan reported is steady state. Even larger transient stimulations of NADH oxidase activity were observed during the first 5 min after mastoparan addition. A homologous but inactive peptide analog of mastoparan, Mas-17, was largely without effect on the steady-state rate of NADH oxidation, nor did its addition induce any transient stimulation.

AlF was tested over the range 0.04 to 40 μM Al^{3+} (0.04 μM Al^{3+} + 0.006 mM F^- to 40 μM Al^{3+} + 6 mM F^-). The mixture stimulated 20% at 4 μM Al^{3+} and 2-fold at 40 μM Al^{3+} (not shown).

Table II. *Stimulation of NADH oxidase of soybean plasma membranes by mastoparan and response to an inactive analog, Mas-17 (Higashijima et al., 1988)*

Results are averages ± SD of three independent determinations.

Peptide	Specific Activity	
	Mastoparan[a]	Mas-17[b]
μM	*nmol min^{-1} mg^{-1} protein*	
0	0.9 ± 0.1	1.0 ± 0.1
1	1.0 ± 0.16	1.0 ± 0.15
5	1.4 ± 0.15	1.1 ± 0.2
10	2.0 ± 0.12	1.0 ± 0.05

[a] Mastoparan, INLKALAALAKKIL. [b] Mas-17, INLKAKAALAKKLL.

Bacterial toxin effects were evaluated using conditions comparable to those used to characterize mammalian G proteins (Ribeiro-Neto et al., 1985; Kahn, 1991). Cholera toxin incubated in the presence of added ADP ribosylation factor reduced NADH oxidase activity when compared with toxin buffer alone but not when compared with no additions (Table III). Toxin buffer (containing 100 μM GTP) reduced 2,4-D responsiveness (not shown), but membranes incubated with toxin buffer plus toxin still supported a 2,4-D response. Pertussis toxin was without effect compared with toxin buffer at the concentration tested (Table III).

GDP also stimulated the NADH oxidase, but the nonmetabolizable GDP analog GDP-β-S did not. Similarly, when

Table III. *Effect of cholera or pertussis toxin on NADH oxidase of plasma membrane from soybean hypocotyl*

Cholera (0.25 μg/μL) or pertussis (0.05 μg/μL) toxins (Sigma) were activated by incubation for 30 min at 30°C in the presence of 25 mM Tris-Mes and 25 mM DTT, pH 7. For ADP ribosylation in the presence of cholera toxin, a mixture of 5 μL of genetically engineered ADP ribosylation factor (0.03 μg/μL, a gift from Dr. Joel Moss, National Institutes of Health), 20 μL of NAD^+ (60 μM), 20 μL of plasma membrane (5 μg/μL), 20 μL of activated toxin, and 60 μL of toxin buffer were incubated on ice for 3 h. At the end of the incubation, NADH oxidase activity was measured. The procedure for pertussis and cholera toxins were similar except that ADP ribosylation factor was not included with pertussis toxin.

Addition	Specific Activity	
	Cholera toxin	Pertussis toxin
	nmol min^{-1} mg^{-1} protein	
No addition	2.3 ± 0.3	1.3 ± 0.7
Toxin buffer[a]	2.9 ± 0.3	1.7 ± 0.5
Activated toxin plus toxin buffer	1.9 ± 0.05	1.7 ± 0.05
+ 10^{-7} M 2,4-D		3.4
+ 10^{-6} M 2,4-D	5.7	5.1
+ 10^{-5} M 2,4-D	7.0	

[a] 300 mM potassium phosphate, 10 mM $MgCl_2$, 10 mM thymidine, 1 mM ATP, and 0.1 mM GTP, pH 7.0.

Table IV. *Response of NADH oxidase of soybean hypocotyl plasma membranes to GTP and GDP-β-S alone and in combination*

Addition	Specific Activity
	nmol min^{-1} mg^{-1} protein
None	1.1 ± 0.2
GDP-β-S (10 μM)	1.1 ± 0.2
GTP (1 μM)	1.4 ± 0.1
GTP (1 μM) + GDP-β-S (10 μM)	1.5 ± 0.1

preincubated with plasma membranes, GDP-β-S prevented GTP stimulation (Table IV). These findings, taken together, suggest that the GDP stimulation of NADH oxidase may have resulted from conversion of GDP to GTP via the enzyme nucleoside diphosphate kinase (see "Discussion").

When plasma membranes were dissolved in Triton X-100 *prior to assay*, NADH oxidase activity was retained at approximately the same or slightly elevated levels as in the absence of Triton X-100 (Fig. 3). The responses to nucleotides GTP, GDP, ATP, and ADP were similar in the presence and absence of 0.1% Triton X-100 (cf. Figs. 2B, 2C, and 3). GTP stimulated at 10 nM but not GDP, ATP, or ADP (Table I). Low concentrations of ATP sometimes appeared to inhibit (e.g. Fig. 1).

Results similar to those observed with guanine and adenine nucleotides were seen as well with uridine, cytidine, and inosine nucleotides (Fig. 4). None of the latter stimulated activity at 10 nM, but UTP, UDP, CTP, and ITP stimulated at 100 nM. All nucleotide di- and triphosphates as well as GMP, guanosine, and guanine stimulated at 1 μM. The latter were without effect, however, at 10 nM or below (Table I). For all the nucleotides tested, the overall form of the dose-response curves showed response proportional to the logarithm of the nucleotide concentration but tended to exhibit a sigmoidal form and a complexity suggestive of multiple nucleotide interactions as nucleotide concentrations were increased.

In the absence of added divalent ions, there was little or no effect of nucleotides on the auxin responsiveness of isolated plasma membranes either freshly prepared (Fig. 5) or after several freeze-thaw cycles (Table V). Comparing GTP and ATP over a range of auxin concentrations as well as GDP and ADP, a 2,4-D response of 1.5- to 1.8-fold was seen at 1 μM (Table V, Fig. 5).

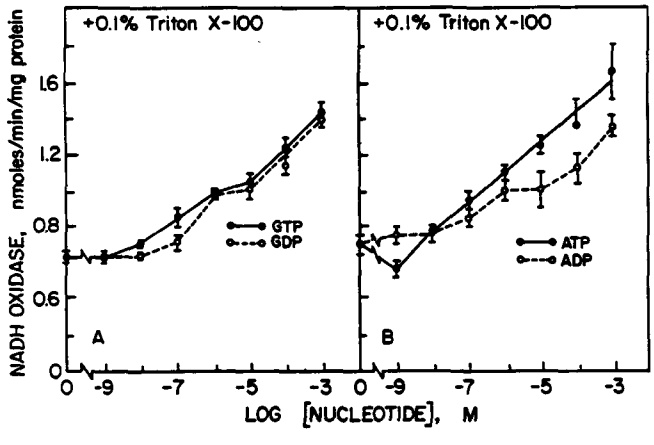

Figure 3. Response of NADH oxidase activity to the nucleotides GTP, GDP, ATP, and ADP in plasma membrane preparations solubilized with 0.1% Triton X-100.

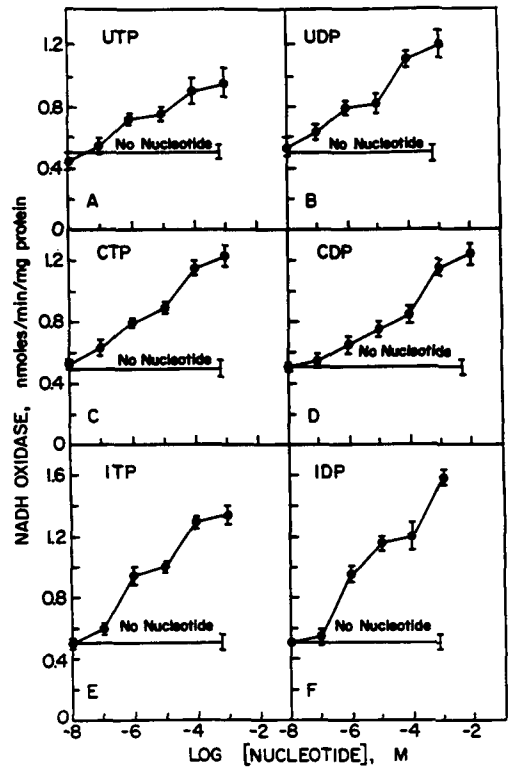

Figure 4. Stimulation of NADH oxidase activity by uridine (A, B), cytidine (C, D), and inosine (E, F) tri- and diphosphates. The rate of NADH oxidation in the absence of nucleotide was 0.5 ± 0.025 nmol min^{-1} mg^{-1} protein.

In the absence of guanine nucleotides, with frozen and thawed plasma membranes, the increment of 2,4-D increase in activity varied from preparation to preparation but was in the range of 0.3 to 0.9 nmol min^{-1} mg^{-1} protein. Even at 20 μM GTP, this same increment of increase due to 2,4-D was maintained (Table V). In the presence of GTP, the 2,4-D response was somewhat less than additive and, with GDP, somewhat more than additive, but for ATP and ADP the 2,4-D and nucleotide responses were nearly additive. This was reflected as well in the V_{max} estimates derived from kinetic analyses (Table VI). For each nucleotide, the increase due to 2,4-D was retained and ranged from 0.3 for GTP to 0.7 for GDP and ADP.

An interaction between nucleotide and 2,4-D was seen, however, in the dose dependency of the response of NADH oxidase to nucleotides measured in the presence and absence of 2,4-D. The affinity was greatest for GTP (Fig. 6) followed by ADP, GDP, and ATP (Fig. 7). With 2,4-D, the apparent concentration of nucleotide for half maximal stimulation of activity was decreased for all four nucleotides (Table VI). The V_{max} with 150 μM NADH was unaffected by nucleotide per se, and the increase reflected only the initial 2,4-D increase

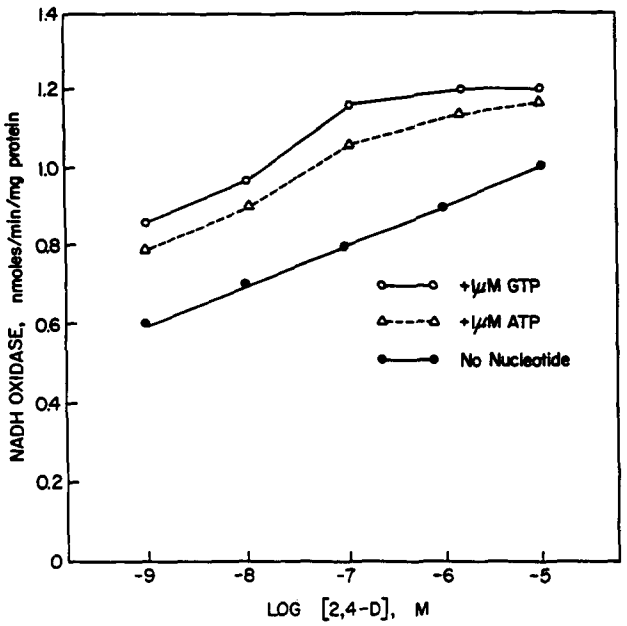

Figure 5. Response of NADH oxidase of soybean plasma membranes, freshly prepared, to increasing concentrations of 2,4-D in the absence (●) and presence of 1 μM GTP (○) or 1 μM ATP (△). The control rate in the absence of 2,4-D or nucleotide was 0.6 nmol min^{-1} mg^{-1} protein. The results are means of duplicate determinations from a single experiment.

(Table VI). The apparent increased affinity for nucleotide was 2.6-fold for GTP and ADP and 4-fold for GDP and ATP (Table VI). For GDP, ADP, and ATP, the form of the curve in the presence of 2,4-D was more nearly hyperbolic, whereas the form of the curve in the absence of 2,4-D was characteristically sigmoidal (Fig. 7).

DISCUSSION

In mammalian cells, G proteins mediate the effects of agonists by working through specific receptors. The final response is the activation or inactivation of some effector protein, which may be an enzyme or an ion channel. Using

Table VI. *Kinetic constants determined for NADH oxidase activity of soybean plasma membrane preparations and response to 2,4-D*

K_s is defined as the concentration of nucleotide for half-maximal stimulation of the NADH oxidase determined by double-reciprocal analysis at an NADH concentration of 150 μM. For K_s determinations, activity in the absence of nucleotide was subtracted. In some instances, e.g. for GTP and ADP, the double-reciprocal plots tended to be sigmoidal, especially with 2,4-D present (Fig. 7), indicative of cooperativity or allostery, but only the linear portions (e.g. 4–20 μM) were utilized for estimation of K_s. V_{max} was determined without subtraction and includes the initial response to 2,4-D.

Nucleotide	K_s		V_{max}	
	No 2,4-D	+10 μM 2,4-D	No 2,4-D	+10 μM 2,4-D
GTP	6.9 ± 3.0	2.7 ± 0.2	2.8 ± 0.2	3.1 ± 0.6
GDP	25.0 ± 4.8	6.6 ± 0.7	2.1 ± 0.1	2.8 ± 0.4
ATP	50.0 ± 9.5	12.5 ± 1.9	2.0 ± 0.8	2.7 ± 0.5
ADP	9.0 ± 1.4	3.8 ± 0.7	2.1 ± 0.2	2.7 ± 0.4

radiolabeled GTP-γ-S, the existence of a small number of GTP-binding proteins has been demonstrated for plants. This evidence is based on GTP-γ-S binding experiments to microsomal (Jacobs et al., 1988; Zaina et al., 1990) or plasma membrane (Blum et al., 1988; Zbell et al., 1990b; Bilushi et al., 1991; Perdue and Lomax, 1992) vesicles from various species, or on partially purified protein fractions (Biffen and Hawke, 1990; Bilushi et al., 1991). A blue light-activated binding of GTP-γ-S has been demonstrated for plasma membranes of etiolated peas (Warpeha et al., 1991, 1992). Here, binding of a nonhydrolyzable photoaffinity-labeling analog of GTP to a 40-kD polypeptide has been observed only after irradiation with blue light. This polypeptide is recognized by polyclonal antisera directed to the α subunit of the G protein transducin, and the polypeptide serves as a substrate for ADP ribosylation by cholera and pertussis toxins. Other evidence comes from using antibodies to synthetic peptides that encompass common sequences contained within animal G_α subunits of the trimeric G proteins (Blum et al., 1988; Jacobs et al., 1988). Subsequent approaches based on molecular cloning have led to the isolation of G_α-like cDNA clones

Table V. *Specific activities of NADH oxidase comparing 2 and 20 μM nucleotide*

Membranes were frozen and thawed several times to enhance 2,4-D responsiveness.

Nucleotide	Concentration	NADH Oxidase		
		No 2,4-D	+ 1 μM 2,4-D	Δ 2,4-D
	μM	*nmol min^{-1} mg^{-1} protein*		
None		0.65 ± 0.05	1.03 ± 0.3	0.4 ± 0.1
GTP	2	0.9 ± 0.025	1.6 ± 0.5	0.7 ± 0.15
	20	2.3 ± 0.4	2.8 ± 0.4	0.5 ± 0.4
GDP	2	0.65 ± 0.05	1.2 ± 0.1	0.55 ± 0.1
	20	1.1 ± 0.1	2.0 ± 0.4	0.9 ± 0.25
ATP	2	0.65 ± 0.05	1.2 ± 0.1	0.55 ± 0.15
	20	1.3 ± 0.1	1.8 ± 0.4	0.5 ± 0.25
ADP	2	0.55 ± 0.05	0.8 ± 0.1	0.25 ± 0.15
	20	0.65 ± 0.05	0.9 ± 0.1	0.25 ± 0.15

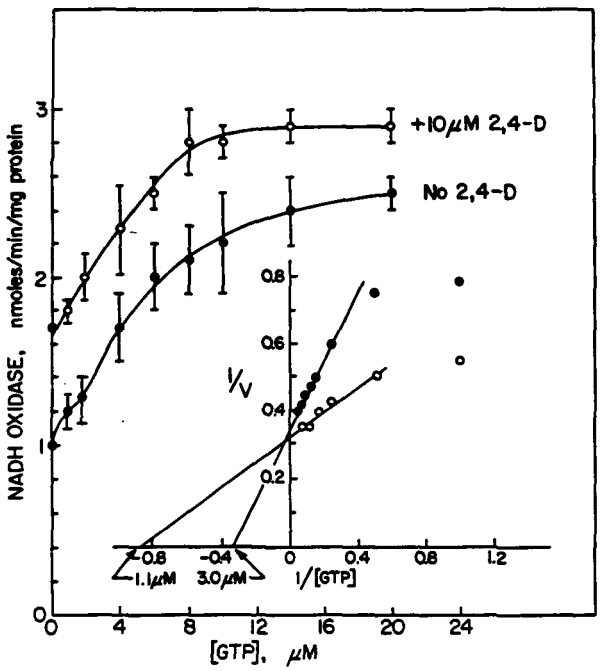

Figure 6. Response of NADH oxidase of soybean plasma membrane activity to increasing concentrations of GTP in the absence (●) or presence (○) of 10 μM 2,4-D. The concentrations for half-maximal stimulation of activity (K_s) for GTP determined from double-reciprocal plots shown were 6.9 μM in the absence of 2,4-D and 2.7 μM in the presence of 2,4-D. Membranes were stored frozen and thawed prior to assay in order to increase 2,4-D responsiveness. Results are based on three experiments, each with duplicate determinations at each time point. Bars indicate ± SD among experiments.

(Blum et al., 1988; Ma et al., 1990, 1991) and of cDNAs encoding low molecular mass GTP-binding proteins of the Ras superfamily (Anaï et al., 1991; Anuntalabhochai et al., 1991; Palme et al., 1992) from plants.

A functional role of GTP-binding proteins in signal transduction has been more difficult to document in plants. A phytochrome-regulated, Ca^{2+}-dependent swelling of wheat protoplasts stimulated by GTP has been reported (Bossen et al., 1990), as has the regulation of the inward K^+ channel current in guard cells of fava bean (Fairley-Grenot and Assmann, 1991). With microsomes of cells of *Acer pseudoplatanus* (Dillenschneider et al., 1986), carrot (Zbell et al., 1990a), or *Dunaliella* (Einspahr et al., 1989), stimulation of PIP_2 breakdown by GTP has been reported, but GTP was without effect on a particulate fraction of PIP_2-phospholipase C (McMurray and Irvine, 1988) or on inositol bis- or triphosphate release from soybean membranes (Biffen and Hawke, 1990).

Among the criteria for involvement of G proteins in physiological responses is that both an appropriate ligand for the receptor of interest and GTP are required to initiate the response in question (Gilman, 1987). Other criteria include induction of a response independently of receptors by inclusion of nonhydrolyzable analogs of GTP (GTP-γ-S) or by AlF_4^- or mastoparan, a 14-residue peptide from wasp venom that activates G proteins by mimicking the region of an activated receptor that normally results in G protein activation (Higashijima et al., 1988, 1990). Mastoparan interacts not only with trimeric G proteins but also with the low molecular mass GTP-binding proteins. Also distinctive is that the α subunits of the trimeric G proteins are substrates for the ADP ribosylations catalyzed by bacterial toxins (Gilman, 1987). Compared with these criteria, some aspects of the NADH oxidase response are atypical for G protein-coupled enzyme activities. Like other plant plasma membranes (e.g. Perdue and Lomax, 1992), soybean plasma membranes exhibit GTPase activity.

Thus, the NADH oxidase of the plant plasma membrane has some characteristics of a G protein-linked activity but differs in other respects from the usual mammalian response. In the absence of added Mg^{2+} ions, the NADH oxidase of soybean membranes was stimulated by low concentrations

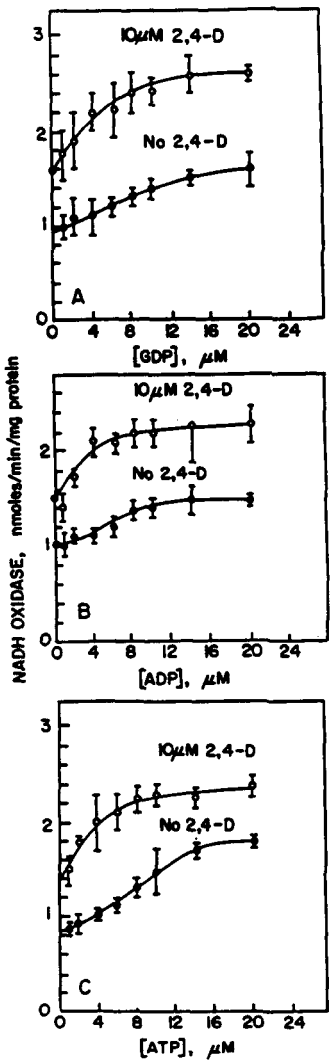

Figure 7. As in Figure 6, the response of NADH oxidase in the presence (○) and absence (●) of 10 μM 2,4-D. Tested were GDP (A), ADP (B), and ATP (C). Results are based on three experiments with duplicate determinations at each time point. Bars indicate ± SD among experiments.

of GTP as well as by the nonhydrolyzable GTP-γ-S. However, GDP did not inhibit but also stimulated the activity, albeit at higher concentrations than GTP. Other nucleotides also were stimulatory but exhibited apparently lower affinities of binding as determined from the response of the NADH oxidase activity to increasing concentrations of nucleotides. It is possible that the latter reflects a general nucleotide-binding activity with lower binding affinity, whereas the GTP effect is more specific and involves principally a high-affinity site.

The concentration differential among stimulatory nucleotides may have relevance in terms of physiological response depending on the relative nucleotide concentrations present at the regulatory site of the responding oxidase. Although GTP, GDP, and other nucleotides all stimulated the activity, the threshold concentrations varied with nucleotide (GTP > ATP > GDP > ADP). This differential was lessened in the presence of auxin, where the concentrations of nucleotides required to stimulate the oxidase were decreased. Zaina et al. (1990) reported increased binding of [^{35}S]GTP-γ-S to membrane vesicles of rice coleoptiles in the presence of the natural auxin indole-3-acetic acid, consistent with these observations.

The stimulation of NADH oxidase by GDP is somewhat problematic but is likely due to the presence in soybean plasma membranes of NDP kinase. This is supported by the observations that NADH oxidase activity in the isolated membranes activated by 2,4-D was stimulated by both GDP and GTP, whereas GDP-β-S, which cannot be phosphorylated by NDP kinase, did not stimulate the oxidase.

NDP kinase catalyzes the transfer of the γ-phosphate of 5′-triphosphate nucleotides to 5′-diphosphate nucleotides via a high-energy phosphorylated enzyme intermediate (Parks and Argawal, 1973). NDP kinase is a ubiquitous activity associated with plasma membranes in addition to the cytosol (Kimura and Shimada, 1990). GDP has been reported to be as effective as GTP in hormone-dependent activation of adenylate cyclase even in the absence of an ATP-regenerating system (Rodbell et al., 1971; Kimura and Nagata, 1977; Kikkawa et al., 1990). These findings have been interpreted as GDP effects that result from the conversion of added GDP into GTP through the action of NDP kinase.

The response of the NADH oxidase activity of the soybean plasma membrane to guanine nucleotides is not that of a usual mammalian G protein-coupled enzyme activity. One explanation for the stimulation by guanine nucleotides is that the NADH oxidase is itself a nucleotide-responsive protein. Guanine nucleotide stimulation was retained in detergent-solubilized membranes, suggesting that interactions afforded by the intact membrane were not required. Also, the observation that 2,4-D treatment modified the affinity for nucleotide binding would be consistent with a direct interaction of nucleotides with the oxidase.

Irrespective of the mechanisms involved, this paper describes a biochemical activity of the plant plasma membrane modulated by guanine nucleotide. It is mastoparan, AlF_4^-, and GTP-γ-S stimulated. These agents constitutively activate G proteins. GTP stimulates, as does GDP, although higher concentrations of GDP are required and GDP-β-S is without effect, suggesting GDP stimulation via conversion to GTP by NDP kinase. Also, GTP and GTP-γ-S stimulated at the nanomolar concentrations at which GDP had no effect. Thus, over a narrow range of concentrations, GTP hydrolysis could be an important mechanism for a return to the basal state following nucleotide stimulation and in the fine control of auxin-regulated growth.

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