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Activation of nucleoside diphosphate kinase by mastoparan, a peptide isolated from wasp venom

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We have previously reported that GDP-bound $\alpha\beta\gamma$-trimeric GTP-binding (G) proteins can be converted into the active GTP-bound form with nucleoside diphosphate (NDP) kinase and ATP, although its exact activation mechanism still remains to be resolved. In the present study, we investigated whether NDP kinase activity was modified by mastoparan, a wasp venom peptide that is known to activate G proteins as an agonist–receptor complex. The activity of NDP kinase measured by the formation of GTP from ATP and GDP was markedly stimulated, when the kinase was incubated with mastoparan. The concentration of mastoparan required for the activation was much lower than that observed for the peptide-induced activation of G proteins under similar assay conditions. There was also an increase in the phosphorylated intermediate of NDP kinase as well as the catalytic activity upon its incubation with mastoparan. These results suggest that mastoparan not only activates G proteins directly via guanine nucleotide exchange reaction but also stimulates NDP kinase activity.

GTP-binding protein; Nucleoside diphosphate kinase; Mastoparan

1. INTRODUCTION

GTP-binding proteins (G proteins) composed of α-, β- and γ-subunits carry signals from membrane receptors to effectors such as enzymes or ion channels [1,2]. Many studies have revealed that dissociation of GDP from the α-subunits appears to be the rate-limiting step in activation of G protein, and this step is accelerated by interaction of G proteins with agonist–receptor complex. It has also been reported that several peptides such as mastoparan, a wasp venom toxin, resemble agonist-bound receptors in their direct interaction with G proteins resulting in activation of the signal-coupling proteins [3–6].

Nucleoside diphosphate (NDP) kinase is a ubiquitous enzyme which catalyzes transfer of the γ-phosphate of nucleoside 5′-triphosphates to nucleoside 5′-diphosphates by a ping-pong mechanism involving transient generation of a high-energy phosphorylated intermediate [7]. It has been observed in several tissues that GDP is as effective as GTP in hormone-dependent activation of adenylate cyclase even in the absence of an ATP-regenerating system [8,9]. This finding suggests that the GDP action may only be manifest as a result of its conversion into GTP by the action of NDP kinase. In addition, ATPγS, an ATP analogue, which can be utilized by NDP kinase as phosphate donor but not bound to G proteins, was reported to trigger G protein-mediated responses in a manner similar to that induced by GTPγS in intact cells or isolated membrane systems [10–12]. Kimura and his colleagues have recently reported that NDP kinase activity is associated with plasma membranes in addition to cell cytosol and that the membrane-associated enzyme may play a role in supplying GTP for the hormone-sensitive adenylate cyclase system [13,14]. NDP kinase may interact with and thus activate G proteins [15], although there is no apparent evidence that GDP bound to G proteins is directly phosphorylated by the kinase [16]. Further investigations into mechanisms whereby the NDP kinase-mediated reactions could be regulated will contribute to exploration of possible roles of NDP kinase in signal transduction processes.

In the present paper, we report that the enzymic activity as well as the phosphorylated intermediate formation of NDP kinase is stimulated by mastoparan. The action of mastoparan on the NDP kinase activity was more striking than its action to stimulate exchange of guanine nucleotides on the α subunit of G proteins.

Abbreviations: G proteins, GTP-binding proteins; ATPγS, adenosine 5′-(3-*O*-thio)triphosphate; GTPγS, guanosine 5′-(3-*O*-thio)triphosphate; DTT, dithiotreitol; SDS, sodium dodecyl sulfate; CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate.

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2. MATERIALS AND METHODS

2.1. *Purification of proteins and reconstitution of G proteins into phospholipid vesicles*

G proteins (G_o) and NDP kinase were purified from cholate extract of bovine brain membranes [17,18] and rat liver cytosol [13], respectively, as described previously. The concentration of the purified G proteins was determined by their abilities to bind [^{35}S]GTPγS as described previously [19]. 200 μl of the purified G_o (300–800 μg of protein/ml) were mixed with 50 μl of purified azolectin (10 mg/ml in 1% Na-cholate) and, after being incubated for 30 min at 0–4°C, were diluted to 350 μl by addition of each 100 μl of 20 mM Na-HEPES buffer (pH 7.4) containing 0.1 mM EDTA and 1 mM DTT. The mixture was filtered through a 15-ml column of Sephadex G-50/fine (Pharmacia-LKB) in the dilution buffer at the flow rate of 0.5 ml/min. Phospholipid vesicles into which G proteins had been incorporated were thus recovered in the voided fraction of the column and stored at −85°C until use.

2.2. *Assays of NDP kinase activity and formation of phosphorylated NDP kinase intermediate*

Indicated concentrations of NDP kinase were incubated with or without mastoparan in 20 mM Na-HEPES buffer (pH 7.4) containing [γ-^{32}P]ATP (0.5 μM; 3 × 10^3 cpm/pmol), 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT and 1 μM GDP at 30°C. At indicated times, the reaction was terminated by addition of an equal volume of an ice-cold solution containing 20 mM Na-EDTA, 1 mM ATP, and 1 mM GTP. The mixtures were spotted on a polyethyleneimine-cellulose thin layer chromatographic plate (Schleicher & Schuell). The plate was immersed into absolute methanol, dried, and then developed with 1.5 M KH_2PO_4 at room temperature. Reaction products were located under an ultraviolet lamp, and the spot corresponding to GTP was counted for the ^{32}P content or quantified by autoradiography to measure the activity of NDP kinase to phosphorylate GDP.

For the purpose of estimation of the [^{32}P]phosphorylated NDP kinase intermediate, NDP kinase (2 μg/ml; 20 nM, if the apparent molecular weight of the kinase is 100,000 Da with a hexamer of 17 kDa subunits) was similarly incubated with indicated concentrations of [γ-^{32}P]ATP (1 × 10^4 cpm/pmol) and mastoparan in the reaction mixture containing no GDP. At indicated times, 20-μl aliquots of the reaction mixture were withdrawn and subjected to the same filtration assay as described in [15]. Another 20-μl aliquots of the mixture were diluted with a SDS-containing buffer, and the radiolabeled kinase intermediate was also analyzed by SDS-polyacrylamide gel (13.5%) electrophoresis and autoradiography.

2.3. *Miscellaneous*

[γ-^{32}P]ATP (1.11 TBq/mmol) and [^{35}S]GTPγS (47.0 TBq/mmol) were purchased from DuPont-New England Nuclear. The sources of all other reagents used were those described previously [17–20]. All experiments were conducted at least twice with different batches of purified proteins, and the results were fully reproducible. Hence, most of the data shown are averages of duplicate determinations with a single batch of experiments that varied within less than 5%.

3. RESULTS AND DISCUSSION

An interaction of mastoparan with NDP kinase was first estimated by measuring the formation of [γ-^{32}P]-GTP from [γ-^{32}P]ATP and GDP. As shown in Fig. 1, the formation of [γ-^{32}P]GTP catalyzed by NDP kinase was stimulated several-fold by mastoparan below its micromolar concentrations. For a comparison, Fig. 1 also illustrates a stimulatory effect of mastoparan on [^{35}S]GTPγS binding to G_o under similar assay conditions. In this case, the half-maximum stimulation of

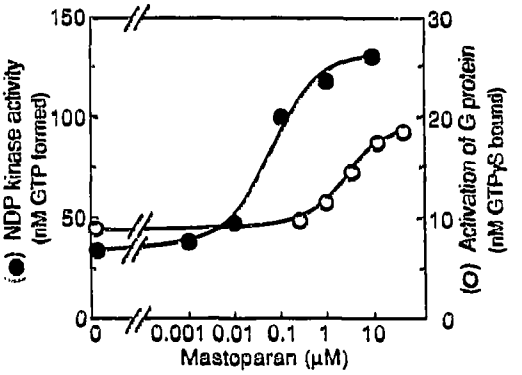

Fig 1. Mastoparan-induced activation of NDP kinase and G proteins. NDP kinase (5 ng/ml; 50 pM) was incubated with 0.5 μM [γ-^{32}P]ATP and 1 μM GDP at 30°C for 6 min in 25 mM Na-HEPES buffer (pH 7.4) containing 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and the indicated concentrations of mastoparan. [γ-^{32}P]GTP formation (●) from [γ-^{32}P]ATP and GDP was determined as described in section 2.2. G_o (30 nM) which had been reconstituted into phospholipid vesicles was also incubated with 2 μM [^{35}S]GTPγS at 30°C for 10 min in the above reaction mixture, and [^{35}S]GTPγS bound to G_o (○) was then measured as described in [19].

[^{35}S]GTPγS binding to G_o was observed with approximately 3 μM mastoparan. Thus the concentration dependencies of mastoparan observed between the two reactions were different from each other. These results suggest that mastoparan exerts its influence not only on the nucleotide exchange reaction of G proteins, as had been reported previously [3–5], but also on the activity of NDP kinase.

Since the NDP kinase activation appeared to be dependent on the formation of phosphorylated intermedi-

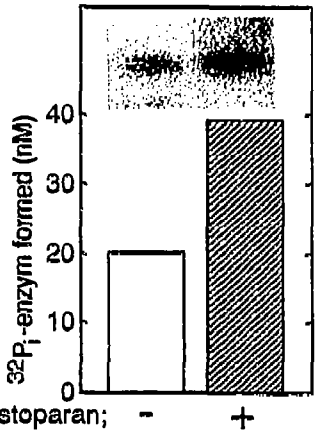

Fig. 2. Generation of phosphorylated NDP kinase intermediate as stimulated by mastoparan. NDP kinase (2 μg/ml; 20 nM) was incubated with or without 30 μM mastoparan at 30°C for 1 min in 20 μl of 25 mM Na-HEPES buffer (pH 7.4) containing 1.1 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM DTT. [γ-^{32}P]ATP (0.5 μM; 1 × 10^4 cpm/pmol) was then added to generate [^{32}P]phosphorylated kinase intermediate during further incubation at 30°C for an additional 1 min. The bars illustrate the result of filter assay while the inset shows the autoradiogram of SDS-polyacrylamide electrophoresis as described in section 2.2.

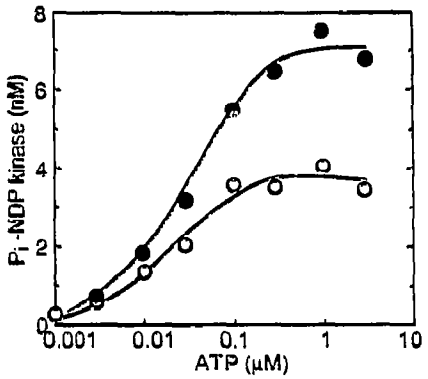

Fig. 3. Stimulation of phosphorylated NDP kinase intermediate by mastoparan under various concentrations of ATP. NDP kinase (500 ng/ml; 5 nM) was incubated with (●) or without (○) 30 μM mastoparan at 30°C for 1 min in 20 μM of 25 mM Na-HEPES buffer (pH 7.4) containing 1.1 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM DTT. [γ-^{32}P]ATP (1×10^4 cpm/pmol) was then added at the indicated final concentrations and followed by the further incubation for 1 min. The amounts of [^{32}P]phosphorylated kinase intermediate were determined as described in section 2.2.

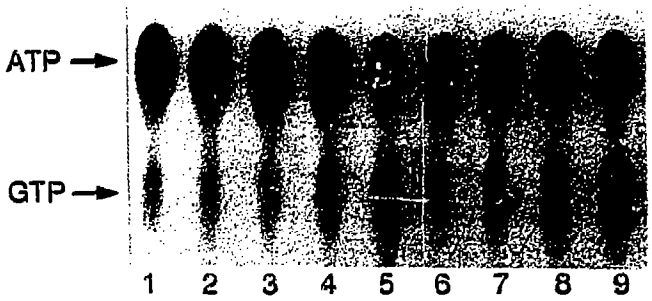

Fig. 4. Stimulation of NDP kinase activity by phospholipids or CHAPS. NDP kinase (5 ng/ml; 50 pM) was incubated with 0.5 μM [γ-^{32}P]ATP and 1 μM GDP at 30°C for 6 min in 25 mM Na-HEPES buffer (pH 7.4) containing 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and the various concentrations of phospholipid (azolectin) or CHAPS. The reaction mixtures, after being diluted with a stop solution, were spotted on a thin layer chromatography plate and then developed as described in section 2.2. The radioactivity of ^{32}P in the fractions of [γ-^{32}P]GTP and [γ-^{32}P]ATP was visualized by autoradiography. (Lane 1) no additives; (lanes 2–5) 1, 10, 50, 300 μg/ml of azolectin, respectively; (lanes 6–9) 0.001, 0.003, 0.01, 0.1% CHAPS, respectively.

ate [7], amounts of the intermediate were also measured by means of SDS-polyacrylamide gel electrophoresis and filtration assay. As shown in Fig. 2, an increase in the [^{32}P]phosphorylated kinase intermediate by mastoparan was confirmed by either of the two assay methods. It may be important to note here that the generation of the phosphorylated intermediate was so rapid and labile that the amount of the intermediate gradually decreased as the incubation time was increased under the present conditions (data not shown).

Fig. 3 shows the effects of various concentrations of ATP, a substrate for the kinase, on the intermediate formation in the presence or absence of mastoparan. The intermediate formation was stimulated by mastoparan to the same extent as all the concentrations of ATP tested; there was no significant change in the apparent affinity for ATP. Mastoparan thus stimulated the catalytic activity of NDP kinase, which was reflected in increased rapid formation of its phosphorylated intermediate without change in the apparent affinity for its phosphate donor.

In addition to mastoparan, several types of phospholipids or detergents were also capable of stimulating NDP kinase activity. Examples are illustrated in Fig. 4. There was an increase in the formation of [γ-^{32}P]GTP from [γ-^{32}P]ATP as the concentration of phospholipid or CHAPS was increased. Other detergents, such as sodium cholate and Lubrol-PX, also enhanced the enzymic activity of NDP kinase, although the enhancement was much less than that observed with CHAPS (data not shown). One of the other G protein activators, compound 48/80 [6], also acted as a stimulator of NDP kinase (not shown).

Our present results indicate that mastoparan exerts its stimulatory effect directly on the catalytic activity of NDP kinase. Such a stimulation of NDP kinase was also observed with certain types of phospholipids or detergents. Since NDP kinase is reported to form a hexamer in the native state [7,13,21], it follows that the enzyme is allowed to form multiple phosphorylated intermediates up to six phosphates per mol of the enzyme. Therefore, it is very likely that the number of phosphorylated subunits is quickly increased by mastoparan, which leads to stimulation of the enzymic activity, without affecting the affinity of the kinase for ATP.

Mastoparan and compound 48/80 are known to mimic certain receptor stimulation in eliciting histamine secretion from mast cells via pertussis toxin-sensitive G protein activation [22]. Certain phospholipids and detergents, too, could activate these G proteins in a cell-free system [23]. The present results showing that these compounds do activate NDP kinase would favor, or would not be at variance with, the idea that NDP kinase-induced phosphorylation of GDP may play a significant role as the supplier of the activator, GTP, to signal transducer G proteins under certain conditions in intact mammalian cells. In any event, the present paper is the first to show a possible regulation of NDP kinase activity by certain agents that mimic receptor stimulation.

We previously reported that NDP kinase was capable of activating $\alpha\beta\gamma$-trimeric G proteins by NDP kinase [15], although no definitive evidence was obtained for the kinase-induced direct phoshorylation of GDP bound to G protein [16]. Quite recently, in a previous paper by Randazzo et al. [24] a report that one of the small GTP-binding proteins, ARF, was directly activated by NDP kinase via phosphorylation of the protein-bound GDP has been corrected by their own stud-

ies*. Thus it is unlikely that NDP kinase can directly phosphorylate GDP bound to GTP-binding proteins.

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