# Characterization of Nucleoside Diphosphokinase Activity in Human and Rodent Pancreatic β Cells: Evidence for Its Role in the Formation of Guanosine Triphosphate, a Permissive Factor for Nutrient-Induced Insulin Secretion†

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ABSTRACT: We have recently demonstrated a permissive role for GTP in nutrient-induced insulin secretion. One of the possible loci at which GTP might exert its regulatory effects include one (or more) of the GTP-binding proteins which we have identified in subcellular fractions (including secretory granules) of pancreatic islets. Herein, we characterize nucleoside diphosphokinase (NDP kinase) activity, which catalyzes the transphosphorylation of nucleotide diphosphate (e.g., GDP) to nucleotide triphosphates (e.g., GTP) in insulin-secreting cells. The presence of NDP kinase activity in normal rat and human islets, and pure β (RIN and HIT) cells, was verified by three distinct approaches: first, its catalytic activity (formation of GTP or GTPγS from GDP and ATP or ATPγS); secondly, by immunologic detection; and third, by quantitating the phosphoenzyme intermediate of NDP kinase, which is involved in a ping-pong phosphotransfer mechanism. Subcellularly, NDP kinase is predominantly cytosolic (with a tetrameric molecular mass of 85–90 kDa) and requires divalent metal ions and thiols for its activity. UDP, which forms an abortive complex with the enzyme, inhibited its activity in a concentration-dependent manner ($K_i$ = 2 mM). The phosphorylated intermediate of NDP kinase was differentially sensitive to heat, acidic pH, and a histidine-selective reagent, diethyl pyrocarbonate, suggesting that (one of) the phosphoamino acid(s) may be histidine. These data demonstrate that in β cells NDP kinase undergoes transient phosphorylation and suggest that this phosphate, in turn, is transferred to GDP. If the GTP which is formed thereby is bound to, or channelled to, relevant GTP-binding proteins, it would facilitate the formation of active form of these proteins. NDP kinase might therefore couple mitochondrial oxidative events to extramitochondrial sites critical to stimulus–secretion coupling.

Glucose-induced insulin secretion from pancreatic islets entails a cascade of events involving the generation of soluble messengers. Some of these factors include ions, adenine nucleotides, cAMP, and lipid hydrolytic products of phospholipase $A_2$, C, and/or D (Prentki & Matschinsky, 1987; Metz, 1986 & 1991). More recently, using selective inhibitors of purine nucleotide synthesis (e.g., mycophenolic acid), we have demonstrated a permissive role for guanosine triphosphate (GTP)[1] in the phenomenon of glucose- and amino acid-induced insulin secretion (Metz et al., 1992). Moreover, by using pathway-specific probes, we have identified the presence of both the "salvage" pathway (using [$^3$H]hypoxanthine as a precursor) and the *de novo* pathway (using [$^{14}$C]glycine as a precursor) in the biosynthesis of guanine nucleotides in normal rat islets; we also demonstrated that glucose can stimulate both of these pathways (Metz et al., 1993). While the exact site(s) at which GTP exerts its modulatory role(s) has not yet been clearly identified, one locus could be one or more low molecular mass and/or heterotrimeric GTP-binding proteins (G-proteins). Recently, we have identified the presence of such proteins enriched in certain subcellular fractions (e.g., secretory granules) of normal rat and human islets and insulin-secreting pure β cell lines (Kowluru & Metz, 1994a–d).

Functionally, the action of low molecular mass G-proteins requires GTP in the GTP-hydrolytic cycle (Bourne, 1988). G-proteins bound to GTP (their active configuration) are converted to their GDP-bound form (inactive configuration) by the GTPase activity intrinsic to G-proteins. These G-proteins reassume their active form either by direct exchange of GDP for GTP (heterotrimeric G-proteins) or via the intermediacy of proteins subserving GDP/GTP exchange activity (Takai et al., 1992). There is a general agreement on the presence and functionality of GDP/GTP exchange activity for low molecular mass G-proteins in many cells (Takai et al., 1992), including pancreatic islets (Kowluru & Metz, 1994b). An additional potential regulatory mechanism might be the conversion of GDP (bound or functionally coupled with G-proteins) to GTP by the enzyme nucleoside diphosphokinase (NDP kinase; NTP:nucleoside diphosphate phosphotransferase; EC 2.7.4.6). NDP kinase catalyzes the transfer of the terminal phosphate from nucleoside triphosphates (NTPs) to nucleoside diphosphate (NDPs) and exhibits broad specificity for the base (Parks & Agarwal, 1973). Most importantly, the transfer of the terminal phosphate occurs by a two-step reaction (also known as the ping-pong mechanism), which was first described by Parks and Agarwal (1973). This

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[1] Abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; ATP, adenosine triphosphate; ATPγS, adenosine 5′-(3-*O*-thio)triphosphate; DEPC, diethylpyrocarbomate; DTT, dithiothreitol; GDP, guanosine diphosphate; GTP, guanosine triphosphate; GTPγS, guanosine 5′-(3-*O*-thio)triphosphate; NDP kinase, nucleoside diphosphate kinase; NDPs, nucleoside diphosphates; NTPs, nucleoside triphosphates; NEM, *n*-ethylmaleimide; PEI, phosphoethyleneimine; PHMB, *p*-hydroxymercuric benzoate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; UDP, uridine diphosphate; UTP, uridine triphosphate.

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involves the formation of a transient high-energy phosphoprotein intermediate form of the enzyme due to phosphorylation of a histidine residue (Parks & Agarwal, 1973; Edlund et al., 1969), followed by transfer of that phosphate to suitable acceptor. There is controversy as to whether NDP kinase can directly convert the GDP-bound form of certain G-proteins to their GTP-liganded form without prior dissociation of GDP from the G-protein in question (Randazzo et al., 1992). However, it has been demonstrated that NDP kinase can convert free (unbound) intracellular GDP to GTP [for reviews, see Parks and Agarwal (1973) and Ray and Mathews (1992)]; this unliganded GTP could then be exchanged with GDP bound to G-proteins, especially if NDP kinase were associated in proximity to the G-proteins or if the GTP formed were to be "channelled" by NDP kinase to a G-protein (Heidbuchel et al., 1993).

There is a growing body of evidence to suggest the involvement of NDP kinase enzyme in several cellular functions. However, very little is known about the presence or functional role of this enzyme in endocrine cells in general and pancreatic islets in particular. Therefore, we undertook the present study to identify its localization, and subcellular distribution, and to characterize this activity in normal rat and human islets. We repeated certain critical studies in insulin-secreting transformed pure $\beta$ cells (HIT or RIN cells) in order to be certain that (at least some of) NDP kinase activity present in islets is attributable to their content of $\beta$ cells.

## EXPERIMENTAL PROCEDURES

*Materials.* [$\gamma$-$^{32}$P]GTP (30 Ci/mmol), [$\gamma$-$^{32}$P]ATP (600 Ci/mmol), and [8-$^{3}$H]GDP (9.7 Ci/mmol) were purchased from NEN-Du Pont (Boston, MA). Nucleoside di- and triphosphates and their nonhydrolyzable analogs (lithium or sodium salts) were obtained from Boehringer Mannheim (Indianapolis, IN). 1,10-Phenanthroline and purified NDP kinase (from Baker's yeast) and creatine kinase (rabbit muscle) were obtained from Sigma Chemical Co. (St. Louis, MO). Affinity-purified polyclonal antiserum for human NDP kinase (nm 23) was obtained from Oncor (Gaithersburgh, MD). This antibody has been shown to recognize human erythrocyte NDP kinase A and B by Western blot analysis (Gilles et al., 1991). PEI-cellulose thin-layer chromatography plates were purchased from E. M Separations (Gibbstown, NJ). Purified $\beta$ subunit of transducin was provided by Dr. Akio Yamazaki, Kresge Eye Institute, Detroit, MI. Mastoparan was purchased from Sigma; mastoparan-17 was from Peninsula Laboratories (Belmont, CA), and mastoparan-7 from Biomol (Plymouth Meeting, PA).

*Pancreatic Islet Isolation and Subcellular Fractionation.* Pancreatic islets were isolated from male Sprague-Dawley rats (300–400 g body weight) by collagenase digestion method as described in Metz et al. (1992). Islets were manually picked twice under stereoscopic control to avoid any contamination by exocrine acinar tissue. Where indicated (Table 2), islets were cultured overnight (18–20 h) in RPMI-1640 medium containing 11.1 mM glucose and other additives as described (Metz et al., 1992, 1993a,b). Human islets (from a total of five donors) were generously provided by Dr. David Scharp, University of Washington Medical School, St. Louis, MO. HIT cells (passage no. 72) were provided by Drs. Paul Robertson and Hui-Jian Zhang, University of Minnesota Medical School, Minneapolis, MN. Purified subcellular fractions of insulinoma cell lines (RIN) were kindly provided by Dr. Chris Rhodes, Joslin Diabetes Center, Boston, MA.

Subcellular fractions from normal rat islets were isolated by the differential centrifugation method as described in Kowluru and Metz (1994a). Details of the purity of these fractions, as determined by marker enzymes as well as by electron microscopy have been described elsewhere (Kowluru et al., 1994). In some experiments, homogenates of normal rat islets as well as of HIT cells were spun at 105000*g* for 90 min (Beckman Ultima TL-100) to obtain total particulate (pellet) and cytosolic (supernatant) fractions (see below). For experiments involving the determinations of kinetic constants for NDP kinase (i.e., the $K_m$, and specificity for various NTPs), homogenates were dialyzed using a microdialyzer (MRA, Clearwater, FL) against 10 mM Tris-HCl, pH 7.4, containing 2 mM DTT at 4 °C overnight (with multiple changes of the buffer) in order to remove the majority of potentially interfering endogenous NTPs, NDPs, and metal ions. For $Zn^{2+}$ chelation experiments, islet cytosol was dialyzed against 50 mM Tris-HCl, pH 7.4, containing of 2 mM DTT and 2 mM phenanthroline (Vallee et al., 1960) at 4 °C overnight with multiple changes of the buffer.

*NDP Kinase Activity.* NDP kinase activity was assayed in homogenates or individual subcellular fractions as described in Seifert et al. (1988) with minor modifications. In brief, the reaction mixture (50 $\mu$L) consisted of 20 mM Tris-HCl, pH 7.5, containing 3 mM DTT, [$^{3}$H]GDP (1 $\mu$Ci/tube), and enzyme protein (5–20 $\mu$g of protein). The reaction was initiated by the addition of unlabeled ATP$\gamma$S or ATP (200 $\mu$M) and was continued for 3 min at 37 °C. It was terminated by the addition of 10 $\mu$L of ice-cold 30 mM sodium EDTA (pH 7.4) and a mixture of unlabeled GDP, GTP, and GTP$\gamma$S (1 mM final concentration) as carrier nucleotides. The tubes were immediately plunged into an ice water bath. An aliquot (10 $\mu$L) of the reaction mixture was applied to PEI-cellulose plate; the nucleotides were separated using 0.75 M $KH_2PO_4$, pH 3.4 as in Kowluru and Metz (1994a). Nucleotides were identified under a UV light (Mineralight UVS-11; UV Products Inc; San Gabriel, CA) using authentic standards, and the radioactivity associated with each spot was quantitated by scintillation spectrometry. In some experiments, NDP kinase activity was also assayed by monitoring the formation of [$\gamma$-$^{32}$P]GTP from [$\gamma$-$^{32}$P]ATP and unlabeled GDP using PEI-cellulose TLC followed by autoradiography.

*Quantitation of Phosphoenzyme Formation.* The phosphorylation reaction was carried out in a total volume of 100 $\mu$L, in a buffer consisting of 50 mM Tris-HCl, pH 7.4, 2 mM DTT, islet protein (up to 30 $\mu$g), and [$\gamma$-$^{32}$P]ATP (1 $\mu$Ci/tube) at 37 °C for different time periods as indicated in the text. It was terminated by the addition of Laemmli stop solution (Laemmli, 1970). Since the phosphohistidine of NDP kinase is heat-sensitive (Wieland et al., 1993), the samples were incubated with the sample buffer at room temperature for 30 min prior to SDS–PAGE. Moreover, it has been recently reported by Wei and Matthews (1991) that typical fixation conditions utilized for SDS gels (i.e, methanol, acetic acid, and water medium) ablates phosphate labeling from [$^{32}$P]phosphohistidine. Therefore, in order to verify that our gel fixation conditions did not underestimate NDP kinase phosphoenzyme formation, gels were fixed in some experiments in 50 mM sodium phosphate buffer, pH 8.0, containing 18.5% formaldehyde for 1.5 h as described in (Wei & Matthews, 1991); these results were compared to those obtained using gels fixed in methanol (40%) and acetic acid (7%) medium. We observed no significant differences in the residual labeling of NDP kinase subunits between these conditions (additional data not shown). Therefore, following separation of proteins on SDS–PAGE (12%; Kowluru & MacDonald, 1984), gels were routinely fixed in methanol–acetic acid medium for 1.5 h and dried at room temperature. Labeled proteins were

identified by autoradiography. Molecular weights of labeled proteins were determined using prestained molecular weight standards (Bio-Rad, Richmond, CA). Labeling intensity of the proteins was quantitated by scanning individual lanes using a Zeineh Video Laser Densitometer (Biomed Instruments, Inc., Fullerton, CA) that was interfaced to an IBM computer equipped with software to calculate the individual peak areas (Kowluru et al, 1994).

*pH and Temperature Stability and Immunoprecipitation of Phosphoenzyme.* Specific phosphoamino acid residues have characteristic patterns of pH and temperature stability (Duclos et al., 1991; Wieland et al., 1993). To determine the stability of phosphoenzyme at acidic and alkaline conditions, islet proteins were phosphorylated as described above and then were incubated with either $H_2O$ (as a control) or 1 N HCl or 1 N NaOH for 30 min at 37 °C. Samples were then neutralized (using either 1 N NaOH or 1 N HCl), and the proteins were separated by SDS–PAGE; the degree of labeling was determined by autoradiography. In order to determine the temperature stability of phosphorylated amino acid(s), samples were incubated either at 25 °C for 30 min (as above) or at 95 °C for 10 min. Proteins were separated by SDS–PAGE, and the degree of residual $^{32}P$ labeling was quantitated by autoradiography as described above.

Immunoprecipitation of the phosphoenzyme from insulin-secreting cell lines was carried out as described in Bominaar et al. (1993). In brief, proteins were phosphorylated as described above, but in the presence additionally of 10 mM NaF, in order to minimize dephosphorylation by phosphatases. The immunoprecipitation sample buffer (consisting of 25 mM Tris-HCl at pH 6.8, 1.5% SDS, 5% glycerol, and 0.25% DTT) was then added to solubilize the proteins. Incubation with anti-NDP kinase (or preimmune serum) was carried out at 4 °C at 1:500 dilution for 2 h. This was followed by an additional incubation (1 h) in the presence of protein A (16 mg/mL) conjugated to agarose beads (Sigma, St. Louis, MO). After incubation, agarose-bound proteins were washed three times in 50 mM Tris, pH 7.4, containing of 150 mM NaCl and identified by autoradiography following SDS–PAGE.

*Detection of NDP Kinase in Insulin-Secreting Cells by Western Blotting.* Proteins were separated by SDS–PAGE and transferred to a nitrocellulose membrane (0.45 μm) using a Bio-Rad transblot apparatus (Metz et al., 1993; Kowluru et al., 1994). Nonspecific protein binding sites were blocked using gelatin. The membranes were then incubated with affinity-purified antiserum raised against NDP kinase (1:1000 dilution) for 15 h at room temperature with gentle shaking. Immune complexes were identified by color development using alkaline phosphatase coupled to anti-rabbit IgG (Bio-Rad, Richmond, CA).

*Immunoneutralization of NDP Kinase Activity in Rat Islet Cytosol.* Islet cytosolic fraction (50–75 μg of protein) was incubated in 50 mM Tris-HCl, pH 7.4, containing 1 mg/ml BSA and 2 mM DTT at 4 °C overnight in the absence or presence of anti-NDP kinase (1:1000 dilution). Residual NDP kinase activity was then assayed (as GTPγS formed) in the presence of EDTA (2 mM) or $MgCl_2$ (3 mM) as described above.

*Determination of the Apparent Molecular Weight of Rat Islet NDP Kinase.* Normal rat islets (1000) were homogenized in a medium consisting of 20 mM Tris-HCl, pH 7.5, containing 2 mM DTT, 0.5 mM phenylmethanesulfonyl fluoride, 10% glycerol, 0.2 mM EDTA, and 50 mM KCl, at 4 °C. The homogenate was then centrifuged at 50000*g* for 20 min, and the supernatant was separated from the pellet. This procedure resulted in separation of more than 90% of the total activity into the supernatant, a finding compatible with observations by Ohtsuki et al. (1986) using HeLa S3 cells. The supernatant was then loaded onto a Sephadex G-200 column (14 × 0.7 cm) column, and 120 μL fractions were collected at 4 °C. The column was calibrated with authentic marker proteins, such as bovine serum albumin (68 kDa), creatinine kinase (from rabbit muscle; 81 kDa), and NDP kinase (Baker's yeast; 85 kDa) to determine the elution volume and the apparent molecular weight of islet NDP kinase. The active fractions were pooled and stored at −70 °C until further use. NDP kinase activity and phosphoenzyme formation were quantitated in each fraction.

*Insulin Secretion.* Insulin secretion was assessed in static (30 or 45 min) incubations of freshly isolated or overnight cultured intact rat islets in Krebs-Ringer bicarbonate buffer containing 3.3 mM glucose and gassed with 95% $O_2$, as described previously (Metz, 1986; Metz et al., 1992, 1993a,b). Insulin was measured by RIA.

*Other Methods.* Protein concentration in samples was assayed according to Bradford using bovine serum albumin as a standard (Bradford, 1976).

## RESULTS

*Optimization of NDP Kinase Activity.* NDP kinase activity was measured in homogenates of normal rat islets by quantitating the formation of [$^3$H]GTPγS from [$^3$H]GDP in the presence of unlabeled ATPγS, or [$^3$H]GTP in the presence of [$^3$H]GDP and ATP. Enzyme activity was linear up to 5 min at 37 °C and proportional to protein concentration of up to 25 μg/assay (additional data not shown). Therefore, NDP kinase measurements were carried out for 3 min using 5–10 μg of enzyme protein. Similar rate constants were observed under these conditions when NDP kinase activity was assayed as the formation of [$^3$H]GTP or [$^3$H]GTPγS (not shown).

*NTP Concentration Dependence and Specificity.* In order to characterize the relative affinities of NDP kinase activity toward its substrates, NDP kinase activity was measured in dialyzed islet homogenates in the presence of increasing concentrations of ATP and ATPγS. Activity was saturable with increasing concentrations of both ATP and ATPγS as phosphate donors; apparent half-maximal velocities were found at 2–3 μM for ATPγS and 15–20 μM for ATP (additional data not shown).

In order to determine whether islet NDP kinase exhibits specificity for NTPs (donor nucleotides), islets homogenates were incubated with ATP, GTP, CTP, UTP, or ITP (200 μM) and [$^3$H]GDP, and the formation of [$^3$H]GTP was studied. Only minimal differences were observed in the activity of NDP kinase supported by various NTPs (range of 6.66–8.51 pmol/min; $n$ = 2 determination with each NTP), suggesting that islet NDP kinase has a broad specificity.

*Divalent Metal Ion Requirement.* NDP kinase activity in other cells typically exhibits a divalent metal ion requirement for optimal activity (Agarwal & Parks, 1971; Parks & Agarwal, 1973). In homogenates of normal rat islets, both $Ca^{2+}$ and $Mg^{2+}$ stimulated the activity in a concentration-dependent manner up to 3 mM (additional data not shown). However, of the divalent metal ions tested, $Mg^{2+}$ stimulated the enzyme activity most potently (up to 17-fold). A similar dose–response relationship between NDP kinase activity and $Mg^{2+}$ was observed in human islet homogenates. In human islet homogenates, $Mg^{2+}$ (1 mM) also stimulated NDP kinase activity (by 8.4-fold). In rat islets, higher concentrations of these ions (above 3 mM) became inhibitory (additional data not shown). The rank order of stimulation of NDP kinase activity was $Mg^{2+}$ (17-fold) > $Mn^{2+}$ (16-fold)

Table 1: NDP Kinase Activity in Insulin-Secreting Cells[a]

| cell type(s) | NDP kinase activity [pmol/(min·mg)] | |
|---|---|---|
| | GTPγS formed | GTP formed |
| rat islets | 175 ± 6 | 294 ± 28 |
| human islets | 104 ± 10 | 440 ± 27 |
| HIT cells | 212 ± 22 | 438 ± 54 |
| RIN cells | 205 ± 5 | 560 ± 16 |

[a] NDP kinase activity was measured in cell homogenates as described in the text. Data represent mean ± SEM of three determinations with each preparation.

> $Ca^{2+}$ (6-fold) > $Ba^{2+}$ (2-fold). These data are consonant with divalent requirements for NDP kinase purified from human erythrocytes (Agarwal & Parks, 1971). $Cu^{2+}$ and $Zn^{2+}$ could not satisfy the requirement for divalent metal ions and, in fact, inhibited the basal activity modestly (up to 20%), but significantly (data not shown). Since pancreatic islets contain significant amounts of $Zn^{2+}$ (Formby et al., 1984), the inhibition of the basal activity by $Zn^{2+}$ prompted us to examine in more detail the effects of this metal ion on maximally stimulated NDP kinase activity (i.e., in the presence of 3 mM $Mg^{2+}$). $Zn^{2+}$ induced a concentration-dependent inhibition of NDP kinase-mediated formation of GTP or GTPγS. Nearly complete inhibition of enzyme activity was seen at 1.5–2.0 mM $Zn^{2+}$. Half-maximal inhibition of enzyme activity (assessed as either GTP or GTPγS formation) was seen at 500–700 μM $Zn^{2+}$. However, inclusion of *o*-phenanthroline (2 mM), a $Zn^{2+}$ chelator, in the assay mixture had no significant effect in the enzyme activity in the cytosolic fraction in the absence of added $Zn^{2+}$ (123 ± 4 pmol/(min·mg of protein) in the control vs 98 ± 10 pmol/(min·mg of protein) in the presence of phenanthroline; $n = 3$ determinations; $p = 0.068$). Furthermore, extensive dialysis of cytosol against *o*-phenanthroline (see Experimental Procedures) had no demonstrable effects on NDP kinase activity in islet cytosol [127 ± 4 pmol/(min·mg of protein) in undialyzed vs 158 ± 18 pmol/(min·mg of protein) in dialyzed cytosol; $n = 3$ determinations; $p = 0.185$).

*Identification of NDP Kinase Activity in Other Insulin-Secreting Cells.* NDP kinase activity was quantitated in homogenates of human islets, rat islets, HIT cells, and RIN cells. Data in Table 1 indicate the formation of GTP and GTPγS in each of the four types of cells studied. GTPγS formation was similar in rat islets, HIT cells, and RIN cells. This activity in human islets, however, represented only 60% of the specific activity seen in rat islet homogenates. GTP formation was found to be similar in human islets, RIN cells, and HIT cells, whereas in rat islets this activity represented only 52% of the activity in RIN cells. The differences in NDP kinase specific activities in these cells could, in part, be due to their content of endogenous NDPs or NTPs (which form potential substrates for NDP kinase activity) or to variations in their GTPase activities (Kowluru et al., 1994). NDP kinase activity measurements have indicated that the transphosphorylation rate of GDP is much higher using ATP as substrate compared to ATPγS (Table 1). These data suggest that the islet NDP kinase possesses a relatively low transthiophosphorylating activity. Similar substrate preference properties have been described for NDP kinases from beef liver (Sheu et al., 1979) and spinach leaves (Nomura et al., 1991).

*Immunological Detection of NDP Kinase in Insulin-Secreting Cells.* Homogenates of rat islets, human islets, RIN cells, and HIT cells were subjected to SDS–PAGE followed by nitrocellulose transfer (see Experimental Procedures). Incubation of nitrocellulose membranes with affinity purified antiserum raised against human NDP kinase indicated that one major protein with an apparent molecular mass of 18–20 kDa was identifiable in all four cell types (Figure 1). However, a second band with an apparent molecular mass of 16–18 kDa was also clearly observed in HIT cells. In human islets, in addition to the 18–20-kDa protein, a protein of ca. 21 kDa reacted with the NDP kinase antisera (Figure 1). These additional bands could represent different isoforms of the NDP kinase subunits, as suggested by a similar pattern of subunits observed during studies of the NDP kinase phosphoenzyme formation (see below).

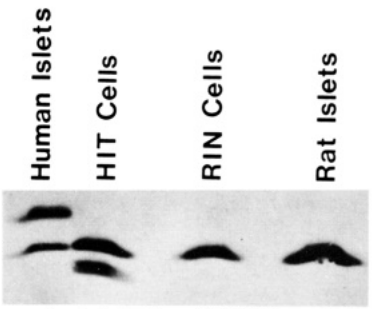


FIGURE 1: Immunologic detection of NDP kinase in insulin-secreting cells. Proteins from homogenates of normal rat islets, human islets, HIT cells, or RIN cells (100 μg of protein) were separated on SDS–PAGE and transferred on to a nitrocellulose membrane. Nitrocellulose membranes were incubated with antiserum directed against human NDP kinase, and the immune complexes were identified using alkaline phosphatase coupled to anti-rabbit IgG (see Experimental Procedures). Molecular weights of proteins were determined using prestained authentic molecular weight standards.

*Subcellular Distribution of NDP Kinase Activity in Rat Islets.* The rank order (expressed as specific activity) for the distribution of this activity in different fractions isolated by differential centrifugation was cytosol > nuclear and plasma membrane > microsomes > mitochondria = secretory granules. Eighty two percent of the total activity in homogenates was recovered in cytosolic fraction (mean of two experiments; data not shown). The predominant cytosolic enrichment of this activity is compatible with the distribution of NDP kinases in other cell types (Parks & Agarwal, 1973). These findings were verified in RIN cells fractions by immunoblotting wherein a predominantly cytosolic distribution of the NDP kinase subunits was observed (additional data not shown).

*Immunoneutralization of NDP Kinase Activity in Rat Islet Cytosol.* To examine whether incubation of islet cytosol (where NDP kinase is predominantly localized) with anti-NDP kinase would result in inhibition of NDP kinase activity, cytosol was incubated with anti-NDP kinase (see Experimental Procedures), and the activity (as GTPγS formed) was assayed. Under these conditions, a modest, but significant (–26 ± 4%; $n = 3$; $p = 0.017$) inhibition of NDP kinase activity was observed.

*Sensitivity to Thiol Group Reagents, UDP, and Mastoparan.* NDP kinase activity in many cells is sensitive to thiol modifying reagents, suggesting the presence of critical sulfhydryl groups in its active site (Mourad & Parks, 1966; Agarwal & Parks, 1971). Either NEM or PHMB (0–100 μM) induced a concentration-dependent inhibition in NDP kinase activity (measured as GTP formed). At 100 μM, NEM and PHMB inhibited the activity by 25% and 80%, respectively (Figure 2A). A higher concentration (500 μM) of NEM inhibited NDP kinase activity by 77%. Moreover, NEM and PHMB (100 μM) inhibited GTPγS formation by 28% and 75%, respectively (Figure 2B), which supports further the conclusion that islet NDP kinase activity requires -SH groups for its activity. A thiol-protective agent, DTT (5 mM), had no effect on either the GTP-forming activity (1.38 ± 0.10

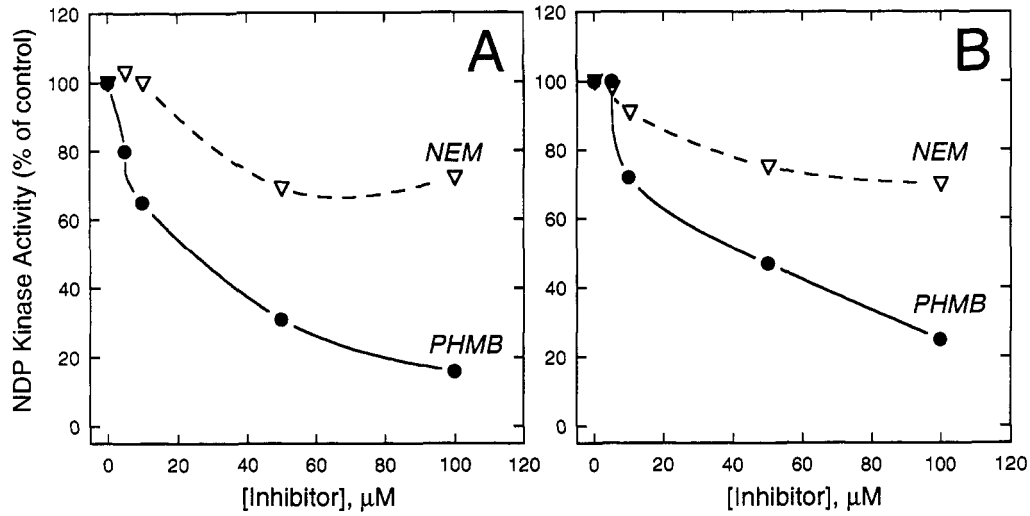


FIGURE 2: Inhibition of NDP kinase activity in islet homogenates by sulfhydryl reagents. NDP kinase activity was assayed in islet homogenates in the presence of increasing concentrations (0–100 μM) of *n*-ethylmaleimide (NEM) or *p*-hydroxymercuribenzoate (PHMB) as described in the text. Activity was measured either as GTP formed (panel A) or GTPγS formed (panel B). Activity measured in the absence of inhibitor was taken as 100%.

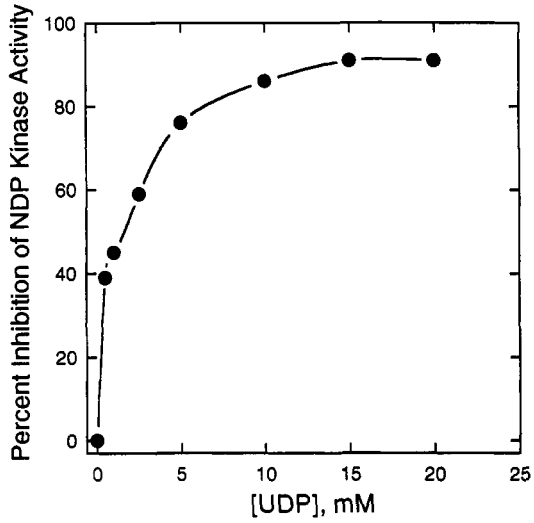


FIGURE 3: Concentration-dependent inhibition of NDP kinase activity in islet cytosol by UDP. NDP kinase activity (quantified as GTPγS formed) was measured in islet cytosol in the presence of increasing concentrations (0–20 mM) of UDP, as described in the text. Percent inhibition of NDP kinase activity as a function of UDP concentration was plotted. Data are representative of two separate experiments with similar results.

pmol/min in control vs 1.24 ± 0.11 pmol/min in DTT treated; $n = 3$ in each case; $p$ = not significant) or the GTPγS forming activity (0.7 ± 0.004 pmol/min in control vs 0.69 ± 0.073 pmol/min; $n = 3$ in each case; $p$ = not significant). Interestingly, however, reduced glutathione (GSH; 5 mM) significantly inhibited both the GTP-forming activity (–43%) and the GTPγS-forming activity (–26%; $n = 3$ in each case; $p < 0.01$).

It has been demonstrated that UDP inhibits NDP kinase activity in liver, erythrocytes, or HeLa cells by forming an abortive complex (Kimura & Shimada, 1983; Jakobs & Wieland, 1989; Parks & Agarwal, 1973). UDP (0–20 mM) inhibited $Mg^{2+}$-stimulated NDP kinase activity in β cells in a concentration-dependent manner, with half-maximal inhibition at 2 mM (Figure 3). UDP (2 mM) also inhibited $Ca^{2+}$-stimulatable NDP kinase activity (–65%; $n = 2$ experiments; $p < 0.01$).

Mastoparan is an activator of GTP-binding proteins (Hagashijima et al., 1990); it has been claimed (Kikkawa et al., 1992) that mastoparan also activates NDP kinase and that this effect might provide one mechanism promoting GTP-for-GDP exchange onto G-proteins. Therefore, the effect of mastoparan on islet NDP kinase activity was examined. Mastoparan stimulated NDP kinase activity in islet homogenates from 1 through 30 μM, with a maximum increase of 43%. Interestingly, however, 30 μM mastoparan-17 (an analog of mastoparan which is inactive on G-proteins; Hagashijima et al., 1990) provoked a similar activation (+54%; data not shown). Furthermore, these increases bore no obvious relationship to the effect of mastoparan to promote insulin secretion, since only mastoparan, but not mastoparan-17, was stimulatory at 30 μM [Table 2; see also Metz et al. (1993b)]. Additional evidence of the dissociation between stimulation of NDP kinase activity and of insulin release could be seen in the fact that 30 μM mastoparan-7 (a "superagonist" of G-proteins) stimulated 247 ± 17% of the amount of insulin released by mastoparan (df. 18; $p < 0.001$) but activated NDP kinase to a generally similar degree (+77%) as mastoparan or mastoparan-17.

*Phosphoenzyme Formation.* Since NDP kinase is typically phosphorylated on a histidine residue involving a ping-pong mechanism (Edlund et al., 1969; Parks & Agarwal, 1973), additional studies of phosphohistidine formation were carried out using cytosol (total soluble) and membrane (total particulate; see Experimental Procedures) fractions. Using [$^{32}$P]ATP[2] as phosphoryl donor, we observed phosphorylation of several proteins[3] in the soluble and particulate fractions (Figure 4A). A protein(s) in the region of 18–20 kDa was phosphorylated in both fractions, presumably representing NDP kinase subunit. Divalent metal ions (e.g., $Mg^{2+}$, $Mn^{2+}$, or $Ca^{2+}$) were required for the phosphorylation of this protein; other metal ions, such as $Zn^{2+}$ and $Cu^{2+}$, failed to stimulate phosphoenzyme formation (additional data not shown), in agreement with our findings of the effects of these metal ions on NDP kinase activity (vide supra).

Further studies were carried out to immunoprecipitate the phosphoenzyme from insulin-secreting cells, to verify that the labeled phosphoproteins did, in fact, represent subunits of NDP kinase. The affinity-purified antiserum directed against NDP kinase precipitated $^{32}$P-labeled NDP kinase subunits

[2] We also observed phosphorylation of NDP kinase subunit(s) in the membrane as well as cytosolic fractions when [γ-$^{32}$P]GTP was used as a phosphoryl donor, thus supporting our findings suggesting a broad NTP specificity for islet NDP kinase (see Results).

Table 2: Effects of Mastoparan or Mastoparan-17 on Insulin Secretion[a]

| | insulin release [$\mu$units/ 10 islets·45 min ($n$)] |
|---|---|
| (A) Freshly Isolated Islets | |
| i. control | 244 ± 32 (3) |
| ii. 30 $\mu$M + mastoparan | 2727 ± 114 (5)[b] |
| iii. 30 $\mu$M + mastoparan-17 | 215 ± 34 (5) |
| (B) Overnight-Cultured Islets | |
| i. control | 92 ± 25 (3) |
| ii. 30 $\mu$M + mastoparan | 438 ± 49 (6)[b] |
| iii. 30 $\mu$M + mastoparan-17 | 89 ± 12 (6) |

[a] Insulin release was assessed in static incubations at 3.3 mM glucose as described under Experimental Procedures. Mastoparan was present only during the 45-min incubation period. Data represent mean ± SEM where ($n$) = number of determinations. [b] $p < 0.001$ vs control.

(Figure 4B) in all four insulin-secreting cells.

*Preliminary Identification of Phosphorylated Amino Acid(s).* Typically, NDP kinase activity involves the formation of an intermediate phosphoenzyme at a histidine site (Parks & Agarwal, 1973; Edlund et al., 1969). Phosphohistidines have characteristic stability, being labile at acidic pH and to heat, unlike most other phosphoamino acids (Wieland, 1993). We took advantage of these properties and studied the stability of putative phosphorylated NDP kinase subunits at 25 and 95 °C (Figure 5A). As a positive control, the stability characteristics of phosphorylated subunits of purified yeast NDP kinase were also examined. Densitometric analyses of the labeled bands indicated that the phosphorylation of NDP kinase subunit from islets, as well as purified yeast NDP kinase, were markedly (50–60%) albeit not totally reduced at 95 °C (Figure 5A). Moreover, we observed that the labeling of NDP kinase subunit was labile at acidic pH (1 N HCl for 30 min at 37 °C) but relatively stable at alkaline pH (1 N NaOH for 30 min at 37 °C), features characteristic of phosphohistidines (Figure 5B; Wieland, 1993). Densitometric analyses of the residual labeling associated with NDP kinase subunit indicated that, under acidic pH conditions, only 20% of the label remained associated with the NDP kinase subunit. Therefore, on the basis of their sensitivities to heat and pH, it seems likely that phosphohistidine comprises most of the phosphoamino acid mass in these proteins; however, some phosphate was probably also incorporated relatively stably into other phosphoamino acids (see below).

These data were extended via histidine site-selective modification. Diethylpyrocarbonate (ethoxy formic anhydride; DEPC) selectively (albeit not with absolute specificity) modifies histidine residues of proteins resulting in the formation of *N*-carbethoxy histidyl derivative (Loosemore & Pratt, 1976; Miles, 1977). This approach has been used for several proteins to elucidate their structure–activity relationships (Miles, 1977). DEPC was used in our experiments to determine its effects on phosphoenzyme formation as well as on NDP kinase activity in whole islets or $\beta$ cells. DEPC inhibited NDP kinase activity in the membrane and cytosolic fractions of HIT cells in a concentration-dependent manner (0–10 mM). At 5–10 mM, DEPC inhibited the GTP$\gamma$S or GTP formation by 74 ± 4% ($n = 6$ determinations; $p < 0.01$). Furthermore, DEPC (10 mM) completely abolished the phosphoenzyme formation in rat islet cytosolic as well as pure yeast NDP kinase (Figure 5C). DEPC also inhibited phosphoenzyme formation in rat islet membrane fraction (data not shown). Together, these data provide evidence that the phosphorylated amino acid may be histidine.

*Determination of Apparent Molecular Weight of Islet NDP Kinase.* The elution pattern of islet cytosolic NDP kinase on a size-exclusion column was used to estimate the apparent

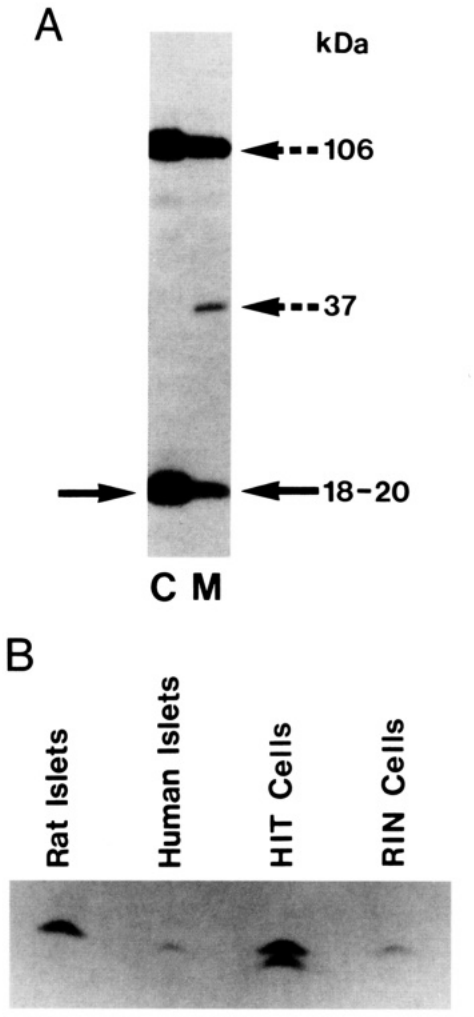


FIGURE 4: (A) Formation of NDP kinase phosphoenzyme in rat islet membrane and cytosolic fractions. Islet membrane and cytosolic fractions were isolated as described under Experimental Procedures and were incubated in the presence of 15–20 $\mu$M [$\gamma$-$^{32}$P]ATP and 3 mM $MgCl_2$ for 3 min at 37 °C. Labeled proteins were separated on SDS–PAGE and identified by autoradiography. NDP kinase subunits (18–20 kDa) were indicated by the solid arrow. Other phosphoproteins (i.e., 37 and 106 kDa) are indicated by broken arrows (see footnote 3). Data are representative of 10–12 experiments with similar results. (B) Immunological identification of phosphorylated NDP kinase subunits in insulin-secreting cells. Homogenates of normal rat islets, human islets, HIT cells, or RIN cells (100 $\mu$g of protein) were phosphorylated as described in the text, in the presence of 3 mM magnesium. Labeled proteins then were incubated with affinity-purified antiserum raised against human NDP kinase followed by incubation of the immune complexes with protein A–agarose. Immune complexes were separated by centrifugation; proteins were separated by SDS–PAGE after extensive washing (see Experimental Procedures). Data are representative of two experiments with similar results.

[3] During our studies of NDP kinase phosphoenzyme formation and of the identification of phosphoamino acid, we observed that a protein with a molecular mass of 37 kDa was also phosphorylated (in the presence of either [$\gamma$-$^{32}$P]ATP or [$\gamma$-$^{32}$P]GTP) in the membrane fraction, but not in the cytosolic fraction, of normal rat islets (see Figure 4A), human islets, HIT cells, or RIN cells (and their insulin-secretory granules). Further functional as well as immunologic studies have indicated that this protein is the $\beta$ subunit of heterotrimeric G-proteins, which we have previously identified in normal human and rat islets and their secretory granules (Kowluru & Metz, 1994a). We also observed that, subsequent to the phosphorylation of a putative histidine residue on the $\beta$ subunit, there was a transfer of the high-energy phosphate via a ping-pong reaction either to free nucleotide diphosphates (e.g., GDP) or GDP-bound to the $\alpha$ subunit of transducin (A. Kowluru and S. A. Metz, manuscript submitted). Wieland et al. (1993) have recently reported the GTP-specific phosphorylation of the $\beta$ subunit of heterotrimeric G-proteins in HeLa cell membranes.

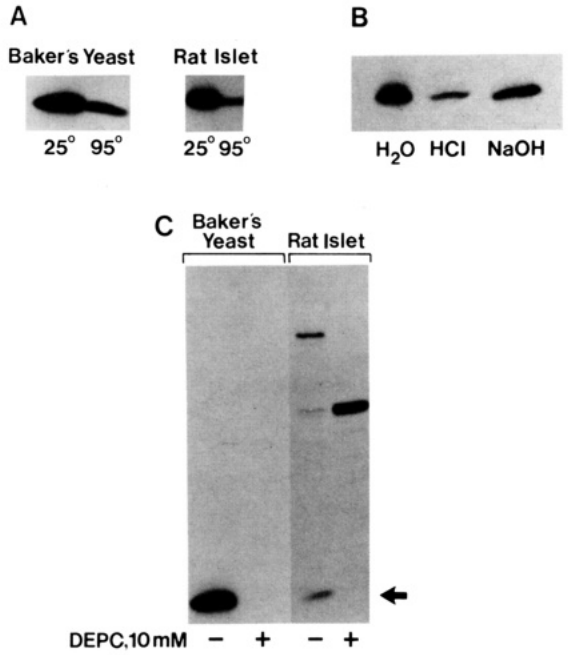


FIGURE 5: Tentative identification of the phosphoamino acid of NDP kinase in islet cytosolic fraction; a comparison to the stability characteristics of Baker's yeast NDP kinase phosphoenzyme. Panel A and B: Islet cytosolic proteins or pure Baker's yeast NDP kinase were phosphorylated as described in the text. Phosphorylated proteins were then incubated either at 25 °C for 30 min or at 95 °C for 10 min and subjected to SDS–PAGE (panel A) or were incubated (rat islet cytosol only) with acid or alkali at room temperature for 30 min and subjected to SDS–PAGE following neutralization (panel B). (Panel C) Islet cytosolic proteins or pure NDP kinase were preincubated at 30 °C for 10 min in the absence or presence of DEPC (10 mM) prior to phosphorylation reaction as described in the text. Labeled proteins were separated by SDS–PAGE and identified by autoradiography. Data are representative of three experiments with similar results.

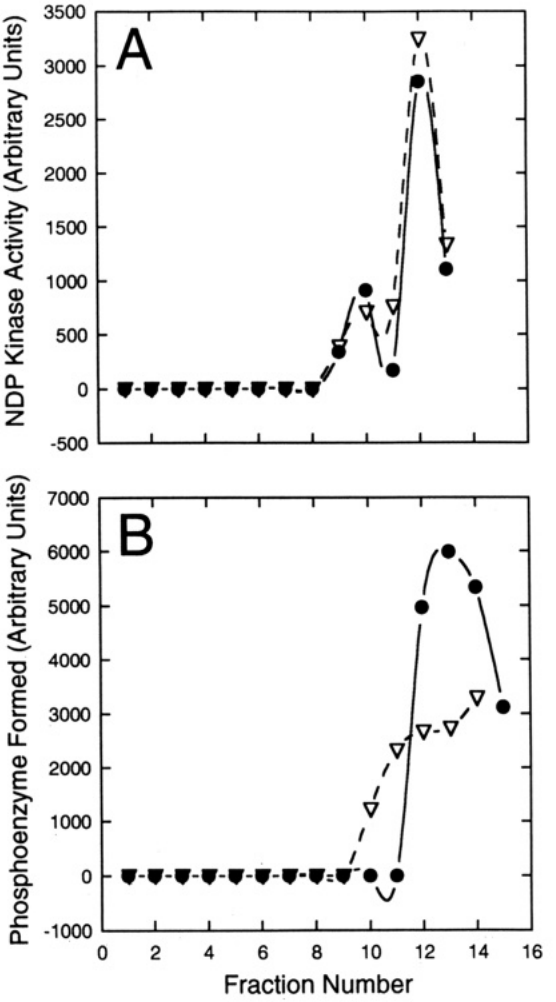


FIGURE 6: Determination of the apparent molecular weight of rat islet NDP kinase activity. Islet homogenates were treated with NDP kinase extraction medium (see Experimental Procedures) and either the islet extract or purified Baker's yeast NDP kinase were loaded on to a precalibrated Sephadex G-200 column; fractions were collected as described in the text. NDP kinase activity (panel A) or phosphoenzyme formation (panel B) were quantitated in each fraction in islets (▽) or pure yeast NDP kinase (●) preparations. Data are representative of two individual column fractionations with similar results.

molecular weight of the islet holoenzyme. Both the islet enzyme and the pure NDP kinase (Baker's yeast) exhibited similar elution profiles with one major peak eluting in the molecular weight region of 85–90K (Figure 6A). Moreover, the pattern of phosphoenzyme formation in these fractions was superimposable on the activity peak (Figure 6B). These findings are compatible with a tetrameric structure for NDP kinase holoenzyme (see below).

## DISCUSSION

One of the goals of the present study was to identify and characterize the subcellular localization of NDP kinase activity in normal pancreatic islets. Although this process is tedious (since 1000 islets comprise only about 500 $\mu$g of protein), we felt that it was critical to characterize the enzyme that catalyzes the conversion of NDPs (i.e., GDP) to NTPs (i.e., GTP) in normal rat islets since we have recently documented a permissive role for GTP in nutrient-induced insulin secretion (Metz et al., 1992). We also repeated critical studies in insulin-secreting pure $\beta$ cells and obtained relatively comparable data using these cell lines. The present findings, therefore, seem attributable (at least to a large degree) to the NDP kinase activity endogenous to the physiologically responsive $\beta$ cell population.

There are remarkably little data available regarding the mechanism of conversion of NDPs to NTPs, the active form of purine nucleotides in islets. While mitochondria convert a substantial degree of ADP to ATP in most cells, there are virtually no extant studies conducted with regard to the conversion of GDP to GTP in insulin-secreting cells. Potential sites for the latter include substrate level phosphorylation via succinylthiokinase in mitochondria (Heldt & Schwalbach, 1967) and NDP kinase in mitochondria (Kleineke et al., 1979) or cytosol. Although the electron-transport chain could in principle use GDP as substrate, GDP is not a substrate for the mitochondrial nucleotide translocase (Heldt, 1966) which is coupled to oxidative phosphorylation. This fact and our earlier studies suggested that cytosolic formation of GTP from GDP may be central in the modulation of insulin secretion (Metz et al., 1992); this formulation stimulated our interest in NDP kinase.

On the basis of the data presented here, one might question whether there is a link between NDP kinase and intracellular GTP levels. Since the intraislet GTP concentrations are rather high (about 0.75 mM), we feel that conversion of GDP to GTP (from GDP and ATP) by NDP kinase may not be required for all GTP-mediated events in islets. However, it seems likely that NDP kinase might play a critical regulatory role in maintaining the GTP/GDP ratio in the "vicinity" of putative G-proteins; such "newly formed" GTP may then be channelled to the G-proteins (Heidbuchel et al., 1993; Haney & Broach, 1994). Moreover, as implicated by Srere (1987), it is also possible that NDP kinase may play a role in coupling

mitochondrial to cytosolic events necessary for insulin exocytosis. This may be achieved by transferring terminal phosphate from mitochondrially derived ATP to (cytosolic) GDP in the proximity of key intracellular organelle(s), i.e., plasma membrane, thus "buffering" a high local GTP/GDP ratio which may be required for insulin exocytosis. In support of this formulation, we would note that our most recent studies suggest that the GTP/GDP ratio might be more important than absolute GTP content in modulating secretion. Furthermore, we observed that maneuvers which reduce mitochondrial ATP formation in islets are accompanied by parallel changes in GTP, UTP, and GTP/GDP ratio, presumably consumed to regenerate ATP via the actions of NDP kinase (M. Meredith and S. A. Metz; manuscript in preparation).

Even though NDP kinase is a logical candidate enzyme subserving such functions, very little information is available on this enzyme in insulin-secreting cells, although Zunkler et al. (1986) have claimed that this enzyme is present in normal rat islets. In the present study, to the best of our knowledge, we have provided the first detailed characterization of NDP kinase activity in peptide hormone-secreting cells. The presence of NDP kinase was verified by three individual approaches: first, its catalytic activity; secondly, by immunologic detection; and third, by quantitating the phosphoenzyme intermediate of NDP kinase phosphorylation at a histidine residue. We observed that NDP kinase activity is predominantly cytosolic in its distribution, compatible with NDP kinases in other tissues. However, some of this activity seems to be associated with the total particulate fraction, albeit to a lesser degree (10–15%); the latter is probably mitochondrial (Kleinke et al., 1979) to a large degree, but some activity seems to be present in secretory granules. In islets, both forms of NDP kinase were similar in their response toward metal ion requirement, inhibitability by UDP, and sensitivities to site-selective reagents such as NEM, PHMB, and DEPC.

$Zn^{2+}$ significantly inhibited the basal, as well as the $Mg^{2+}$-stimulatable NDP kinase activity, as well as the formation of its phosphoenzyme intermediate, with half-maximal inhibition at ca. 500–750 $\mu$M $Zn^{2+}$. It is well documented that islet subcellular fractions (especially the secretory granules) contain significant (mM) amounts of $Zn^{2+}$ (Formby et al., 1984). However, $Zn^{2+}$ chelation experiments indicated that sufficient amounts of $Zn^{2+}$ are apparently not present (or bioavailable) to inactivate the islet NDP kinase, since no significant effects on cytosolic NDP kinase activity were demonstrable in the presence of *o*-phenanthroline. Thus, $Zn^{2+}$ may not physiologically regulate cytosolic NDP kinase activity *in situ*, possibly due to sequestration of the former. Both the catalytic activity, as well as the phosphoenzyme formation of NDP kinase exhibited a clear requirement for divalent metal ions. However, in interpreting these data, we have not taken into consideration any differential effects of these metal ions (e.g., $Mg^{2+}$ or $Mn^{2+}$) on protein phosphatases, GTPases, or the transfer of phosphate from phosphohistidine to acceptor molecules (Kowluru & Metz, 1994d; Goldenring et al., 1986; Barik, 1993; Wong et al., 1993).

On the basis of the elution characteristics on a Sephadex G-200 column of the NDP kinase from normal rat islets, the intact islet enzyme has an apparent molecular mass of 85–90 kDa, similar to the mass of the Baker's yeast enzyme. Data from immunoblotting and the phosphoenzyme studies have indicated the presence of two subunits of NDP kinase in the molecular mass range of 18–20 kDa. Therefore it is likely that the islet NDP kinase may exist as tetrameric enzyme with a subunit size of 18–20 kDa. Similar oligomeric subunit composition has been widely reported for NDP kinases in literature (Parks & Agarwal, 1973).

On the basis of differential sensitivities to acidic pH, to temperature, and to DEPC, we inferred that majority of the phosphoamino acid mass in the cytosolic and the membrane forms of islet NDP kinase is phosphohistidine. These findings are compatible with data on other NDP kinases from different tissues in which it was documented that the His-118 residue is the site of phosphorylation for the ping-pong reaction (Gilles, 1991). However, the pH and temperature stability characteristics of phosphoamino acids in NDP kinase phosphoenzyme suggested that, in addition to histidine, there might be additional, less abundant phosphorylation sites in NDP kinase, since neither acid nor heat totally obliterated the labeling. Data from earlier studies have provided evidence for additional phosphorylation of NDP kinases at sites which are acid- and alkali-resistant (Hossler & Rendi, 1971). As suggested by Parks and Agarwal (1971), such NDP kinases might be subserving functions in the cell different from the transfer of high-energy phosphates to nucleotide diphosphates. However, we can not presently rule out the possibility that NDP kinase can be phosphorylated on sites in addition to histidine. Indeed, MacDonald et al. (1993) have recently reported phosphorylation at a serine residue of metastasis suppressor protein Nm23, a protein which also has an intrinsic NDP kinase activity; it is possible that such phosphorylation could modulate the activation of NDP kinase. It is also possible that NDP kinase comigrates precisely with another protein which is phosphorylated at an amino acid other than histidine.

Recent studies by Vu and Wagner (1993) have indirectly implicated NDP kinase activity in ATP$\gamma$S-induced noradrenaline secretion from PC12 cells. These investigators proposed that NDP kinase in PC12 cells converts ATP$\gamma$S to GTP$\gamma$S, which then activates a putative G-protein(s) responsible for noradrenaline secretion. The present studies provide direct evidence for the presence, subcellular distribution, and characterization of NDP kinase activity in four insulin-secreting cells. A finding that NDP kinase can be regulated by modulators of insulin secretion would help to place this enzyme in the cascade of events leading to insulin secretion. One such modulator might be mastoparan. It has been reported recently that mastoparan, a tetradecapeptide from wasp venom (Hagashijima et al., 1990), stimulates the catalytic as well as phosphoenzyme formation of NDP kinase in rat liver cytosol (Kikkawa et al., 1992). Therefore, it is of interest that studies from several laboratories (Jones et al., 1993; Komatsu et al., 1993), including our own (current studies; Metz et al., 1993b) have demonstrated that mastoparan stimulates insulin secretion from isolated rat islets as well as from pure $\beta$ cell lines by an unidentified mechanism. Furthermore, we observed that this peptide, in insulinotropic concentrations (30 $\mu$M), stimulated NDP kinase activity in islets. However, mastoparan-17, an analog of mastoparan which is inactive on G-proteins (Hagashijima et al., 1990) and which did not exert stimulatory effects on insulin secretion, nonetheless did stimulate NDP kinase activity to a degree comparable to mastoparan itself (current studies and A. Kowluru, S. E. Seavey, M. E. Rabaglia, and S. A. Metz, manuscript submitted). Therefore, it seems likely that the stimulatory effects of mastoparan analogs on NDP kinase activity is nonspecific, and largely unrelated to their effects on insulin release. In fact, the effects of mastoparan-17 (which augmented NDPK activity but not insulin release) suggest that an activation of NDP kinase may be an insufficient stimulus by itself to stimulate the $\beta$ cell secretory machinery. However, these initial studies do not, of course, exclude a role

for NDP kinase in the insulin secretion induced by physiologic agonists.

We have also recently obtained evidence that insulinotropic fatty acids, such as arachidonic acid (Metz, 1986, 1991; Wolf et al., 1986), also stimulate both the phosphoenzyme formation and the catalytic activity of NDP kinase in islets and HIT cells (Kowluru & Metz, 1994d). Thus, these data may identify a locus (namely, the NDP kinase activity) by which lipids exert their modulatory effects on islet G-protein function (Kowluru & Metz, 1994a–c; Kowluru et al., 1994), and could provide a possible novel mechanism whereby this activation might culminate in insulin secretion.

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