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Short Communication

NON-SPECIFIC STIMULATORY EFFECTS OF MASTOPARAN ON PANCREATIC ISLET NUCLEOSIDE DIPHOSPHOKINASE ACTIVITY: DISSOCIATION FROM INSULIN SECRETION

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Abstract—We examined whether mastoparan (MAS)-induced insulin secretion might involve the activation of nucleoside diphosphokinase (NDP kinase), which catalyzes the conversion of GDP to GTP, a known permissive factor for insulin secretion. MAS and MAS 7 (which activate GTP-binding proteins), but not MAS 17 (an inactive analog), stimulated insulin secretion from normal rat islets. In contrast to their specific effects on insulin secretion, MAS, MAS 7 and MAS 17 each stimulated formation of the phosphoenzyme-intermediate of NDP kinase, as well as its catalytic activity. These effects were mimicked by several cationic drugs. Thus, caution is indicated in using MAS to study cellular regulation, since some of its effects appear to be non-specific, and may be due, in part, to its amphiphilic, cationic nature.

Key words: pancreatic β cells; insulin secretion; mastoparan; nucleoside diphosphokinase; GTP-binding proteins; amphiphilic cationic drugs

MAS†, an amphiphilic tetradecapeptide from wasp venom, has been shown to exert a variety of biological effects, including stimulation of secretion from mast cells [1], chromaffin cells [2], normal rat pancreatic islets and insulin-secreting RINm5F cells [3–5]. MAS has been shown to activate several enzymes *in vitro*, such as phospholipase A_2 [6] and adenyl cyclase [7]. Most of these effects of MAS have been attributed, at least in part, to its ability to activate both low molecular mass as well as heterotrimeric GBPs [8, 9]. One mechanism by which MAS could activate GBPs would be to activate NDP kinase (EC 2.7.4.6), which catalyzes the conversion of nucleotide diphosphates (e.g. GDP) to nucleotide triphosphates [e.g. GTP; Ref. 10]. Recently, we characterized NDP kinase in soluble fractions of normal rat islets, human islets, and pure β (RIN and HIT) cells [11]. The GTP generated by NDP kinase may have important functional effects in insulin-secreting cells. We also have documented a permissive role for GTP in nutrient-induced insulin secretion using selective inhibitors of purine nucleotide biosynthesis [12]. Therefore, in the present study we asked whether MAS-induced insulin secretion from pancreatic islets might involve activation of islet NDP kinase. These studies were stimulated, in part, by the recent observations by Kikkawa *et al.* [10], who reported activation by MAS of NDP kinase activity purified from rat liver cytosol. We also examined the effects of MAS 7, a more potent analog of MAS, and MAS 17, an analog of MAS with no effect on GBPs [8], on NDP kinase activity and on insulin secretion, to determine whether their effects on insulin secretion were a specific reflection of their effects on NDP kinase activity (i.e. formation of phosphoenzyme-intermediate as well as catalytic activity).

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† Abbreviations: ATPγS, adenosine 5′-[γ-thio]triphosphate; BAC, benzoylalkonium chloride; DTT, dithiothreitol; GTPγS, guanosine 5′-[γ-thio]triphosphate; GBPs, GTP-binding proteins; MAS, mastoparan; NDP kinase, nucleoside diphosphokinase; PTx, pertussis toxin.

Materials and Methods

[γ-^{32}P]GTP (30 Ci/mmol), [γ-^{32}P] ATP (600 Ci/mmol), and [^{3}H] GDP (9.7 Ci/mmol) were purchased from NEN-Dupont (Boston, MA). ATP, GTP, ATPγS, and GTPγS were obtained from Boehringer Mannheim (Indianpolis, IN). MAS and BAC were purchased from Sigma (St. Louis, MO). MAS 17 was from Peninsula Laboratories (Belmont, CA). MAS 7 and compound 48/80 were obtained from Biomol (Plymouth, Meeting, PA).

Isolation of pancreatic islets for studies of insulin release. Pancreatic islets were isolated from male Sprague–Dawley rats (2–4 months of age; 300–400 g body weight) by the collagenase digestion method as described previously [3, 11–13]. Insulin secretion was assessed in 30-min static 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_2/5% CO_2, as described previously [3, 11–13]. Mastoparan or its analogs were present in concentrations as indicated in the text. Insulin was quantitated by radioimmunoassay.

NDP kinase assay. For NDP kinase assay [11], islets were homogenized in 50 mM Tris–HCl, (pH 7.4) containing 1 mM DTT and 2.5 μg/mL each of leupeptin and pepstatin. In some studies, islets were homogenized manually (0.75 to 1 mg protein/mL) in a glass homogenizer using an isotonic buffer consisting of 230 mM mannitol, 70 mM sucrose and 5 mM HEPES (pH 7.4) and were then centrifuged at 105,000 *g* for 60 min to obtain the cytosolic fraction (supernatant). The NDP kinase assay mixture (50 μL) consisted of 20 mM Tris-HCl, (pH 7.5) containing

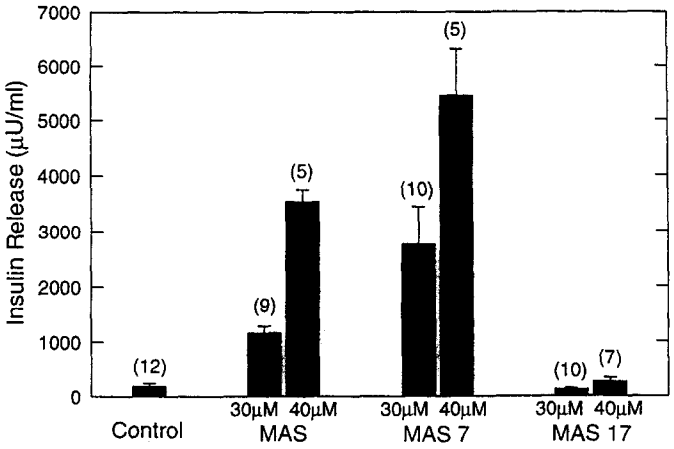

Fig. 1. Effects of MAS and its analogs on insulin secretion from normal rat islets. Insulin release was measured at 3.3 mM glucose in the absence or presence of MAS, MAS 17, or MAS 7 (at 30 or 40 μM) under static incubation conditions for 30 min. Data are from two independent experiments and represent means (± SEM) of individual islet incubations (number of determinations indicated in parentheses) at each experimental condition. Statistical significance: $P < 0.001$ for MAS or MAS 7 vs control; $P < 0.001$ for MAS vs MAS 7. Data demonstrating the efficacy of MAS and the lack of effect of MAS 17 were confirmed in four independent experiments overall.

3 mM DTT, 3 mM $MgCl_2$, [^{3}H]GDP (1 μCi/tube) and islet protein (5–20 μg). The reaction was initiated by the addition of unlabeled ATPγS (200 μM), and was continued for 3 min at 37°. These incubation conditions were found to be optimal for NDP kinase activity in islet homogenates. The reaction was terminated by the addition of 10 μL of ice-cold 30 mM sodium-EDTA (pH 7.4), and a mixture of unlabeled GTP, GDP, and GTPγS (1 mM final concentration) was added as carrier nucleotides. An aliquot (10 μL) of the reaction mixture was applied to the PEI TLC plate (E.M. Separations, Gibbstown, NJ), and the nucleotides were separated using 0.75 M KH_2PO_4 (pH 3.4), as described previously [11, 13]. Nucleotides were identified under a UV light using authentic standards, and the radioactivity associated with each spot was quantitated by scintillation spectrometry.

Quantitation of NDP kinase phosphoenzyme formation. This was carried out in a total volume of 50 μL, in a buffer consisting of 50 mM Tris–HCl (pH 7.4), 2 mM DTT, 3 mM $MgCl_2$ (where needed), islet protein (up to 30 μg) and [γ-^{32}P]ATP (1 μCi/tube) at 37° for 2 min. The reaction was terminated by the addition of Laemmli stop solution. Since the phosphohistidine of NDP kinase is heat-sensitive [11], the samples were incubated in the sample buffer at room temperature for 30 min prior to SDS-PAGE. To avoid the loss of label from phosphohistidine under acidic conditions, following separation of proteins on SDS-PAGE, gels were fixed briefly (1.5 hr) in methanol–acetic acid medium and dried at room temperature, and the degree of labeling was quantitated by densitometry of the autoradiograms [11].

Results and Discussion

MAS (30 μM) stimulated insulin secretion by 8.7-fold at a non-stimulatory glucose concentration of 3.3 mM (Fig. 1). Its insulinotropic effect was more pronounced (>19-fold) at 40 μM. In contrast, MAS 17, which is an inactive analog of MAS [8], failed to elicit any effect on insulin secretion (even up to 40 μM), suggesting that the effects of MAS are specific (Fig. 1). MAS 7, a MAS analog with 5-fold greater potency than MAS in stimulating purified GBP functions (i.e. GTP binding and hydrolysis; [8]), augmented insulin secretion by 18.4- and 30-fold (Fig. 1) at 30 and 40 μM, respectively. The effect of MAS 7 on insulin secretion was significantly more potent than that of MAS (Fig. 1). Taken together, these data suggest that the effects of MAS analogs on insulin secretion are related to a stimulation of GBPs. Indeed, we previously reported that the insulinotropic effect of MAS was blunted (−63%) by PTx pretreatment of islets to inactivate heterotrimeric GBPs [3].

We next sought to determine whether this stimulation of insulin secretion was related to NDP kinase activation, presumably acting on a pool of GDP which is coupled to GBPs. Indeed, MAS stimulated the NDP kinase activity in islet homogenates in a concentration-dependent manner. Maximal activation (up to 43%) was seen at 30 μM MAS (Fig. 2A). We then studied the effects of MAS analogs on NDP kinase activity. Data in Fig. 2B indicate that MAS 7 (30 μM) also stimulated NDP kinase activity up to 77%. However, to our surprise, MAS 17 (30 μM) stimulated NDP kinase activity as potently as MAS itself (+54%). These data indicate that both active and inactive analogs of MAS stimulate NDP kinase activity. We, therefore, asked whether the MAS 17 effect reflected non-specifically its cationic, membrane-perturbing properties. We examined this possibility by studying the effects of other polycationic compounds that perturb lipid bilayers, such as compound 48/80 [14], and observed that compound 48/80 stimulated the NDP kinase activity in islet homogenates by 23% (Fig. 2B).

The activation of NDP kinase involves the *transient* formation of a high-energy phosphohistidine-intermediate; this phosphate, in turn, is transferred to nucleotide diphosphate(s) to yield nucleotide triphosphate(s) via a ping-pong mechanism [15]. We recently described such a mechanism in islets; we observed the phosphorylation of an 18–20 kDa protein (in normal rat islets, human islets, and insulin-secreting pure β cells), which was immunoprecipitated by NDP kinase antiserum [11]. In 1992, Kikkawa *et al.* [10] reported stimulation by MAS of rat liver cytosolic NDP kinase phosphoenzyme formation. In the present study, we examined the effects of MAS (or its analogs) on NDP kinase phosphoenzyme formation in islet cytosol. A consistent, albeit variable (20–78%;

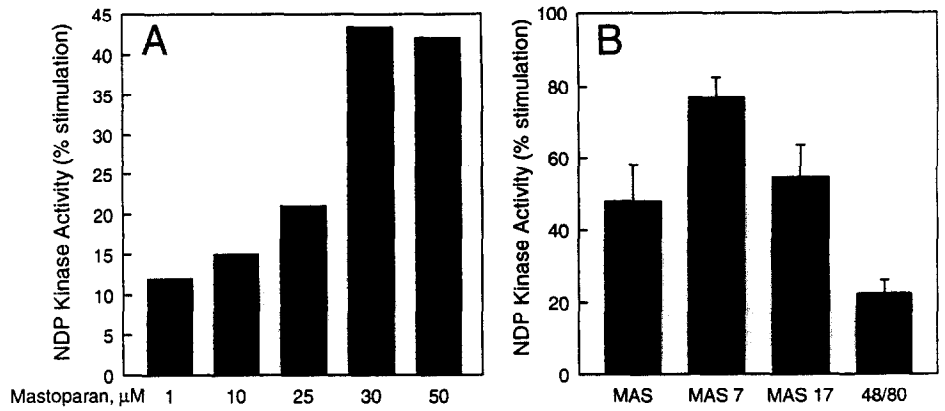

Fig. 2. Stimulation of NDP kinase activity in rat islet homogenates by MAS, MAS 7, MAS 17, or compound 48/80. (A) NDP kinase activity was assayed in the presence of various concentrations of MAS as indicated in the figure. Data represent the means of 2–4 individual incubations at each concentration. (B) NDP kinase activity was assayed in the absence or presence of MAS, MAS 7, MAS 17, or compound 48/80 (at 30 μM). Data represent the means (± SEM) of 3–5 individual incubations at each condition. MAS, MAS 17, MAS 7, or compound 48/80 significantly ($P < 0.01$) stimulated NDP kinase activity compared with control (no drug). Data are expressed as percent stimulation (activity observed over the activity measured in the absence of drug [taken as 100%], which represented 175 ± 6 pmol of [^{3}H]GTPγS formed per min per mg protein).

N = 3 determinations) stimulatory effect of MAS on phosphoenzyme formation was observed in islet cytosol. Similar phosphorylation of NDP kinase was seen in the presence of MAS 7 or MAS 17 (50–75%; N = 2 determinations each). In addition, another poly-cationic drug, BAC (20 μg/mL), significantly increased the NDP kinase phosphoenzyme formation (+211%; N = 2 determinations) whether provided alone or in combination with MAS (30 μM).

The exact mechanism(s) underlying the insulinotropic effects of MAS is unknown [3–5]. Recent studies by Jones *et al.* [4] and Komatsu *et al.* [5] have excluded the possibility that MAS-induced insulin secretion involves the activation of protein kinase C or A or the stimulation of calcium influx. Rather, its effects probably involve the activation of one (or more) GBPs (a possibility in accord with the current studies). Data from our recent studies [3] indicate that MAS-induced insulin secretion may involve a PTx-sensitive mechanism. There are extant data to suggest that cationic amphiphilic agents, such as MAS or compound 48/80, activate PTx-sensitive G-proteins (i.e. G_i and G_o) in a receptor-independent manner [1, 8, 14]. MAS activates GBPs directly in a manner similar to the effects of receptor occupancy induced by extracellular agonists [8]. One of the suggested mechanisms for such an activation is the ability to act as substitutes for the third cytoplasmic loop of receptors, which has been implicated in GBP activation [8]. It has been shown that PTx irreversibly ribosylates a cysteine residue near the C-terminus of the α subunits of heterotrimeric GBPs, resulting in inhibition of the GBP stimulatory effects of mastoparan [8, 12]. The observed effects of mastoparan analogs on insulin secretion have the same rank order of potency as their ability to activate GBPs (i.e. MAS-7 > MAS > > MAS-17). Thus, these effects are not believed to be non-specific pharmacologic or structural effects. Recent studies by Vitale *et al.* [2] have shown that MAS, but not MAS 17, induces exocytosis of catecholamines from chromaffin cells, suggesting a critical structural specificity in these peptides to promote exocytosis. Alternatively, the activation of GBPs could involve stimulation of NDP kinase, which could "channel" the GTP formed to relevant GBPs [11, 16]. While MAS does activate NDP kinase and thereby converts GDP to GTP, the concomitant effects on insulin secretion may not involve the intermediacy of NDP kinase activation, since MAS 17 stimulated NDP kinase activity (but not secretion) significantly.

Our present findings should prompt investigators to include suitable controls (such as MAS 17) in their studies. We note that such controls are conspicuously absent in all previous extant studies dealing with the effects of MAS either on insulin release or NDP kinase activity. Caution should be used when MAS is provided as a probe for GBPs, since some of its effects appear to be non-specific, and may be due, in part, to the amphiphilic, cationic nature of these peptides [8] acting in a manner similar to divalent metal ions [11]. This possibility is further supported by the fact that BAC and compound 48/80, which are poly-cationic drugs, exerted similar stimulatory effects on NDP kinase. This caveat may extend to many other frequently used cationic pharmacologic probes, such as melittin, mepacrine, or tetracaine.

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