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Gastric Inhibitory Polypeptide Activates MAP Kinase through the Wortmannin-Sensitive and -Insensitive Pathways

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The signal transduction pathways of a cloned human gastric inhibitory polypeptide (GIP) receptor have been investigated in CHO cells stably expressing this receptor. Exposure of GIP receptor expressing cells to GIP significantly increased MAP kinase activity. Time course analysis showed that a rapid and marked increase in MAP kinase activation was detected and that this activation reached maximal levels 10 min after the addition of GIP. Dose-response analysis showed that GIP activated MAP kinase activity in a dose-dependent manner with an ED_{50} value of 5.9×10^{-10} M of GIP. Wortmannin, a potent inhibitor of phosphatidylinositol 3-kinase (PI3-kinase), partially inhibited GIP-induced MAP kinase activation, suggesting that GIP activates MAP kinase through two different, wortmannin-sensitive and -insensitive pathways. It has been demonstrated that in CHO cells cAMP attenuates MAP kinase activity by inhibiting Raf-1. Since GIP elevates intracellular cAMP, we examined the effects of cAMP on MAP kinase activation. Interestingly, forskolin, which increased intracellular cAMP levels, significantly inhibited MAP kinase activation by GIP, but did not affect MAP kinase activation by GIP in the presence of wortmannin, suggesting that the wortmannin-sensitive pathway activates an MAP kinase cascade at or above the level of Raf-1 and that the wortmannin-insensitive pathway activates an MAP kinase cascade below the level of Raf-1. These findings demonstrate that the GIP receptor is linked to the MAP kinase cascade via at least two different pathways.

GIP is a 42-amino acid peptide hormone belonging to the vasoactive intestinal peptide (VIP)/glucagon/secretin family (1-4). It was first isolated from porcine small intestine on the basis of its ability to inhibit gastric acid secretion (1). Subsequent studies, however, of the physiological role of GIP demonstrated that GIP potently stimulates insulin secretion in the presence of elevated glucose as an incretin (5,6). Thus, GIP is now referred to as glucose-dependent insulinotropic polypeptide.

GIP exerts its biological effects by binding to its specific high-affinity receptors (7,8). It has been demonstrated using insulinoma cell lines and isolated islets that GIP stimulates insulin secretion by increasing intracellular cAMP and Ca^{2+} concentration (9-13). We have recently isolated human GIP receptor cDNA (14), which has seven putative transmembrane domain, a characteristic feature of G protein-coupled receptors. We have established Chinese hamster ovary (CHO) cells stably expressing the GIP receptor (CHO-GIPR) (15), which enables us to elucidate the signal transduction pathways of this receptor in a reconstituted system. Mitogen-activated protein (MAP) kinase comprises a family of serine/threonine kinases activated by growth factors, hormones and neurotransmitters (16-20). MAP kinase is a key enzyme in the regulation of cellular proliferation, differentiation, and metabolism. Recent studies show that secretagogues such as glucose, GLP-1, and PACAP stimulate MAP kinase activity (21), suggesting the involvement of MAP kinase cascade in pancreatic β cell function. However, whether or not GIP affects MAP kinase cascade is not yet known.

On the other hand, recent study showed that GIP and VIP/PACAP stimulate insulin secretion through a cAMP-independent and wortmannin-sensitive pathway (22,23), but the nature of this signalling pathway is not known.

In the present study, we have investigated the possible cross talk of GIP signaling with MAP kinase cascade and the effect of wortmannin on this pathway. Here we report that GIP stimulates MAP kinase activ-

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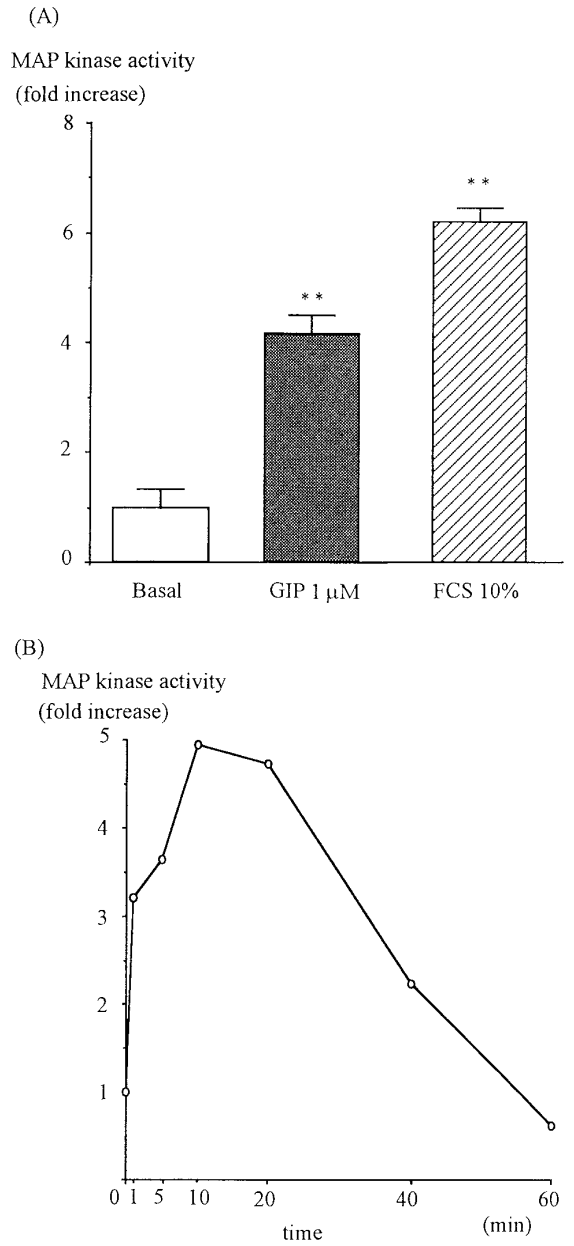

FIG. 1. Effects of GIP on MAP kinase activity in CHO-GIPR. (A) CHO cells were incubated with 1 μM GIP or 10% fetal calf serum for 5 min and MAP kinase activity was determined. Data are means ± S.E. of triplicate determinations. **p<0.01 vs control. (B) CHO cells were incubated with 1 μM GIP for the indicated times and MAP kinase activity was determined.

ity in CHO cells stably expressing the GIP receptor and that this pathway is partially inhibited by wortmannin.

MATERIALS AND METHODS

Measurement of MAP kinase activity. CHO cells were grown in monolayers in α minimal essential medium (αMEM, GIBCO/BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) in 5% CO_2 at 37 °C.

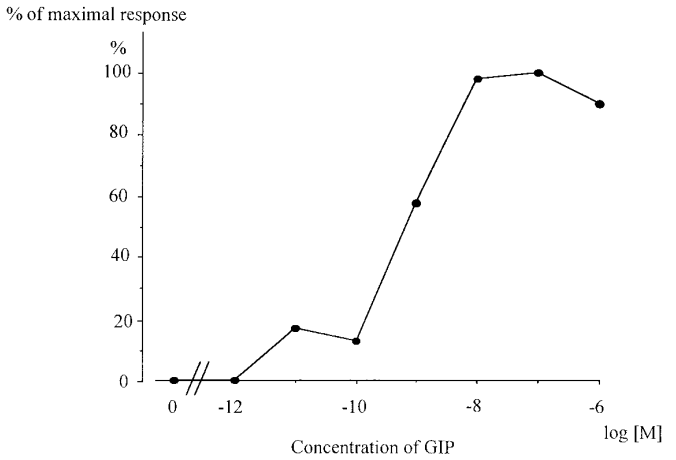

FIG. 2. Dose-response analysis of GIP-induced MAP kinase activation. CHO cells were incubated with varying concentrations of GIP for 5 min and MAP kinase activity was determined.

CHO cells stably expressing human GIP receptor (15) were grown to 50 % confluency in 10 cm culture dishes and the culture medium was changed to αMEM containing 0.01%BSA (fatty acid-free) and incubated for 24 h before the assays. The reaction was started by adding the ligands at the indicated concentration and after 5 min incubation at 37 °C, the cells were washed two times with ice-cold phosphate buffered saline and quick-frozen in a liquid nitrogen bath. The cells were scraped and homogenized in the buffer containing 20 mM Tris (pH7.4), 2 mM EDTA, 20 mM β-glycerophosphate, 1 mM vanadate, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 2500 KIE/ml aprotinin and crude cell lysate was recovered as a

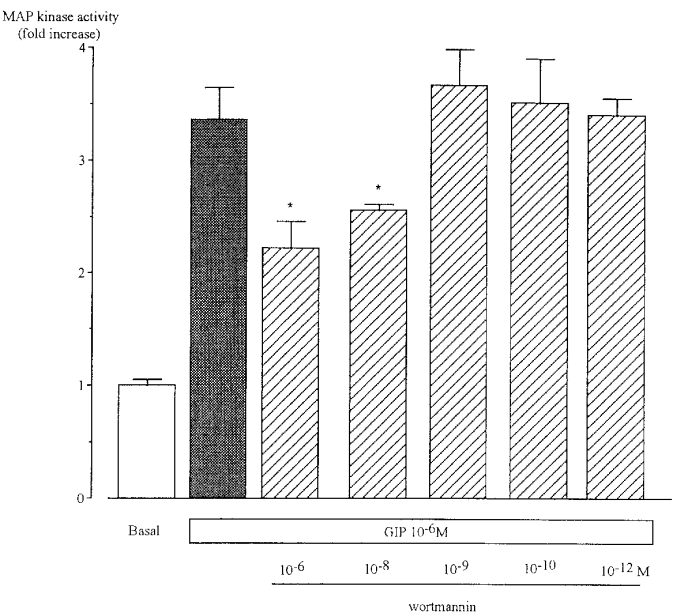

FIG. 3. Effects of wortmannin on GIP-induced MAP kinase activation. CHO cells were preincubated with wortmannin at the indicated concentration and incubated with 1 μM GIP in the presence of wortmannin and MAP kinase activity was determined. Data are means ± S.E. of triplicate determinations. *p<0.05 vs GIP-(without wortmannin) stimulated level.

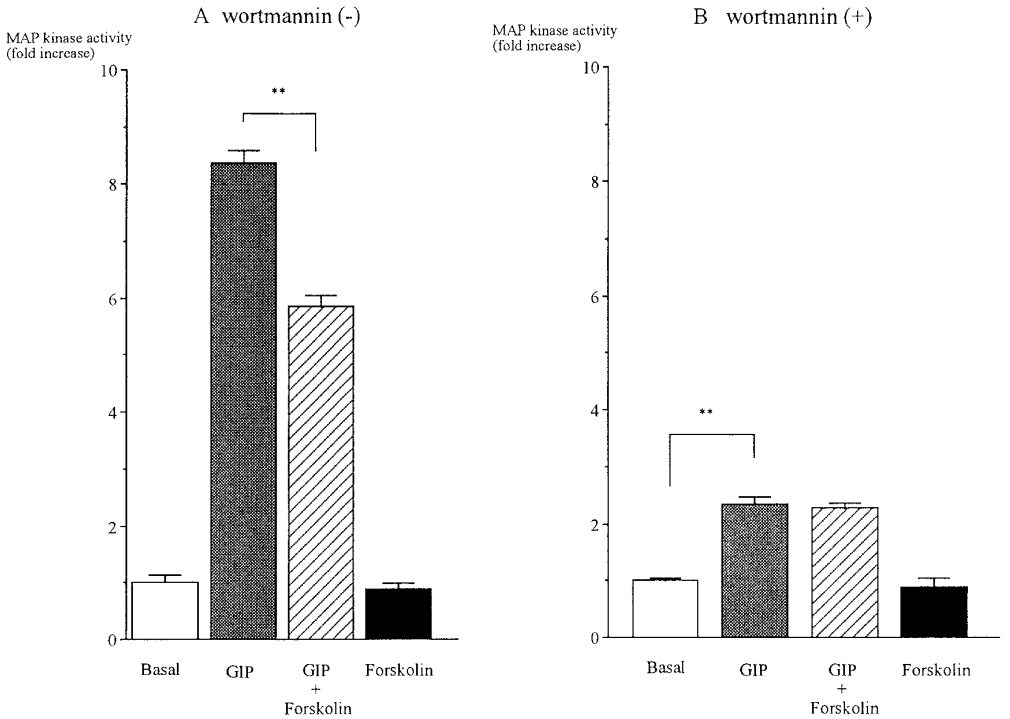

FIG. 4. Effects of forskolin on GIP-induced MAP kinase activation with (A) or without (B) wortmannin. (A) CHO cells were incubated with 1 μM GIP and/or 50 μM forskolin and MAP kinase activity was determined. (B) CHO cells were preincubated with 10 nM wortmannin and incubated with 1 μM GIP and/or 50 μM forskolin, and MAP kinase activity was determined. Data are means ± S.E. of triplicate determinations. **$p<0.01$.

supernatant after 140000 x g centrifugation for 25 min at 4 °C. The MAP kinase activities of the samples were measured using a MAP kinase assay kit (Amersham). Wortmannin was donated by Kyowa Hakko Kogyo (Tokyo, Japan).

Measurements of cAMP formation. Measurement of cAMP formation was carried out essentially as described previously (24). Briefly, CHO cells were grown to confluency in 12-well plates. The cells were incubated with 500 μl of Krebs-Ringer buffer containing 1 mM 3-isobutyl-1-methylxanthine (IBMX) with or without GIP (Peptide Institute, Osaka, Japan) at the indicated concentration at 37 °C for 5 min. The reaction was stopped by adding 100 μl of 30% trichloroacetic acid and the cAMP levels were determined by radioimmunoassay kit (Yamasa, Chiba, Japan).

RESULTS AND DISCUSSION

In our previous report, GIP increased cAMP levels in CHO-GIPR, indicating that the GIP receptor is functionally expressed in the cell line (15). Using this cell line, we investigated the possible involvement of MAP kinase cascade in the pathways of signal transduction elicited by GIP. In non-transfected CHO cells, GIP did not affect MAP kinase activity (data not shown). Exposure of GIP receptor expressing cells to GIP led to a 4.2 fold increase in MAP kinase activity (Fig.1A). By time course analysis, a rapid and marked increase in MAP kinase activation was detected, which reached maximal levels 10 min after the addition of GIP (Fig.1B). Dose-response analysis showed that GIP activated MAP kinase activity in a dose-dependent manner with an ED_{50} value of 5.9×10^{-10} M of GIP (Fig.2). These results demonstrate that GIP receptor is linked to MAP kinase cascade.

Because it has been reported that several G protein-coupled receptors are linked to MAP kinase cascade through a wortmannin, a potent PI3-kinase inhibitor-(25,26) sensitive pathway (27-29), we have investigated the effect of wortmannin on GIP-induced MAP kinase activation. The kinase activity was measured after a 30-min preincubation of the cells with wortmannin. Wortmannin dose-dependently inhibited GIP-induced MAP kinase activation with maximal inhibition at a concentration of as low as 10^{-8} M wortmannin (Fig.3). These results indicate that PI3-kinase is involved in GIP-induced MAP kinase activation. Furthermore, the inhibition of MAP kinase activation by wortmannin was partial, suggesting the existence of wortmannin-insensitive pathway in the GIP-induced MAP kinase activation. In our previous report, GIP elevated intracellular cAMP levels in CHO cells expressing the GIP receptor. Since it has been reported that cAMP inhibits MAP kinase in CHO cells (30-32), we examined the effect of cAMP on GIP-induced MAP kinase activity. While forskolin which elevated intracellular cAMP did not affect basal MAP kinase activity in CHO cells, it siginificantly inhibited GIP-induced MAP kinase acti-

TABLE 1

Intracellular cAMP Levels of CHO-GIPR

	Basal	GIP 1 μM (pmol/well)	GIP 1 μM+forskolin 50 μM
Wortmannin (−)	0.288 ± 0.05	6.62 ± 0.38	75.4 ± 4.61
Wortmannin 10 nM	0.267 ± 0.08	6.28 ± 0.25	73.5 ± 2.85

Note. CHO cells were incubated with GIP or with GIP and forskolin for 5 min and cAMP levels were determined. Data are means ± S.E. of triplicate determinations.

vation (Fig.4A). It has been reported that cAMP inhibits MAP kinase cascade at the level of Raf-1 (33). These results suggest that the wortmannin sensitive pathway activates MAP kinase cascade at or above the level of Raf-1. Our results are consistent with the report that signalling from G protein-coupled receptor to MAP kinase via PI3-kinase require sequential interaction of Shc, Grb2, Sos, Ras, Raf and MAP kinase kinase (34). Interestingly, forskolin did not inhibit GIP-induced MAP kinase activation in the presence of wortmannin (Fig.4B), suggesting that the wortmannin-insensitive pathway activates MAP kinase cascade below the level of Raf-1. It was confirmed that wortmannin did not affect GIP-induced cAMP formation (Table 1). Although several laboratories reported the existence of Raf-independent pathway for the activation of MAP kinase (35-37), there is no report of Raf-independent pathway which is linked to G protein-coupled receptors. Therefore our results first suggest the existence of Raf-indepndent pathway from G protein-coupled receptor to MAP kinase. However the detail of the pathway is not known and still requires further study.

Recent study has demonstrated that a wortmannin-sensitive signalling pathway other than elevated cAMP and [Ca^{2+}] is involved in GIP-induced insulin secretion (22). However, the wortmannin-sensitive pathway is not known. Therefore, since MAP kinase activation by GIP is blocked by wortmannin, MAP kinase cascade is a candidate for wortmannin-sensitive signalling involved in peptide hormone-induced insulin secretion.

In summary, GIP stimulates MAP kinase activity in CHO cells expressing the GIP receptor through at least two different, wortmannin-sensitive and -insensitive pathways. Although the physiological significance of MAP kinase activation by GIP remains to be clarified, these results provide a better understanding of the mechanism of GIP action.

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