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AN INHIBITORY ROLE FOR PHOSPHATIDYLINOSITOL 3-KINASE IN INSULIN SECRETION FROM PANCREATIC B CELL LINE MIN6

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SUMMARY: Phosphatidylinositol 3-kinase (PI3-kinase) has been implicated in the regulation of vesicular transport. We examined the roles of PI3-kinase in the glucose-induced insulin secretion from the pancreatic β cell line MIN6 by using wortmannin, a potent inhibitor of PI3-kinase. Low concentrations of wortmannin markedly potentiated the glucose-induced insulin secretion. This effect was probably mediated by PI3-kinase inhibition. Furthermore, wortmannin completely canceled the suppressive effect of insulin-like growth factor-I on insulin secretion from MIN6 cells. On the basis of these results, we discuss a possible role of PI3-kinase in the negative feed-back regulation of insulin secretion.

A series of intracellular signaling events induced by the interaction of cell surface receptor tyrosine kinases with their ligands include the stimulation of protein tyrosine kinase activity and the activation of enzymes including phospholipase C and PI kinases (1). The activity of PI3-kinase is found to exist in a complex of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit (2, 3), and is associated with many protein tyrosine kinases (4, 5). Detailed analysis of the mutant protein tyrosine

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Abbreviations: PI, phosphatidylinositol; IGF, insulin-like growth factor; MLCK, myosin light chain kinase; KRBB, Krebs-Ringer bicarbonate buffer; EDTA, ethylenediaminetetraacetic acid; IRI, immunoreactive insulin; RBL, rat basophilic leukemia.

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kinases has suggested the importance of their association with PI3-kinase activity in intracellular signaling. Insulin and IGF-I also initiate their biological effects by the activation of their receptors containing protein tyrosine kinase activity. The activated receptors phosphorylate IRS-1, a high molecular-weight cytoplasmic protein, which binds to and activates PI3-kinase (6).

The major physiological stimulus for insulin secretion from pancreatic β cells is glucose, but the levels of insulin secretion are modified by a number of hormones, among which insulin, IGF-I, and somatostatin exert an inhibitory action on the insulin release. The inhibitory effects by insulin and IGF-I have been shown to be mediated by their binding to the IGF-I receptors on β cells (7), although the precise mechanisms for this regulation including the role of PI3-kinase remain to be elucidated.

Recently, we and others have found that wortmannin, a microbial product, which is known as an MLCK inhibitor (IC_{50} = several hundreds nM) (8), is a very potent inhibitor of PI3-kinase (IC_{50} = several nM) (9-11). These reports have also suggested that PI3-kinase plays an important role in the regulation of protein trafficking. In the present study, we have examined the roles of PI3-kinase in the insulin secretion from the glucose-responsive β cell line MIN6 (12) using wortmannin.

MATERIALS AND METHODS

Reagents: Wortmannin was prepared from the fermentation broth of *Talaromyces wortmannii* KY12420 as described (8). Somatostatin and recombinant IGF-I were purchased from Peptide Institute Inc. (Osaka, Japan) and Upstate Biotechnology, Inc. (Lake Placid, NY), respectively.
Cell cultures: MIN6 cells were cultured as previously described (12). The cells used in the present study were from passages 11-27.
Insulin secretion: MIN6 cells were seeded at 2 x 10^5 cells/well in 24-well culture plates. Two days later, the cells were washed twice with KRBB (119 mM NaCl, 4.7 mM KCl, 2.54 mM $CaCl_2$, 1.19 mM $MgSO_4$, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM HEPES buffer, pH 7.2, and 0.2% BSA), and preincubated in KRBB containing 5 mM glucose at 37 C for 15 min. Then, various concentrations of wortmannin dissolved in dimethyl sulfoxide was added to the culture (final 0.1 v/v% dimethyl sulfoxide), and the cells were incubated for another 15 min. In some experiments, 10 nM of somatostatin or IGF-I were added 10 min after addition of wortmannin. After washing with KRBB, further incubation was performed in 1 ml KRBB containing the same constituents with those before washing but various concentrations of glucose for 90 min. At the end of the incubation period, the buffer was collected, centrifuged briefly, and the supernatants were assayed for mouse insulin by enzyme-linked immunosorbent assay (ELISA) using rat insulin as a standard.

PI3-kinase activity assay: MIN6 cells were seeded in 6-well culture plates. Two days after plating, the cells were washed twice with KRBB and treated with various concentrations of wortmannin for 15 min as described above. The cells were then stimulated with 20 mM glucose for 15 min in the presence of wortmannin, and washed twice with KRBB containing 20 mM glucose. The PI3-kinase activity was assayed as described (9).
Immunoprecipitation and immunoblotting: Lysates of MIN6 cells treated with wortmannin were prepared as described above. The cell lysates were incubated with anti-PI3-kinase p85 serum or anti-wortmannin monoclonal antibody for 1 h and then with protein G-Sepharose beads for 1 h. The beads were washed, and proteins bound to the beads were eluted with SDS sample buffer and separated by SDS-PAGE. After transfer onto nitrocellulose membrane, resolved proteins were detected with anti-wortmannin antibody or anti-PI3-kinase serum and appropriate alkaline phosphatase-conjugated second antibody followed by 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.
Statistical analysis: Statistical significance was determined using the Student's t-test or one-way ANOVA followed by the Post-hoc Dunnet's procedure.

RESULTS

Effects of wortmannin on insulin secretion from MIN6 cells

We first examined the effects of different concentrations of wortmannin on the insulin secretion from MIN6 cells (Fig. 1). Wortmannin at concentrations ranging from 5 to 50 nM markedly enhanced insulin release at 15 mM glucose. Such stimulatory effects of wortmannin were dose-dependent within this range. At 50 nM of wortmannin, the insulin release was 3.5-fold higher than that without this drug. The apparent ED_{50} of wortmannin for this stimulation was estimated to be ~8 nM. However, the insulin release began to decrease beyond this range of wortmannin, and 10 μM of wortmannin strongly inhibited the insulin secretion. Since wortmannin has been shown to inhibit MLCK with an IC_{50} of several hundred nM in the previous works (9), the suppression of insulin secretion by wortmannin was probably due to the inhibition of MLCK. This result suggests that myosin-actin interaction through the activation of MLCK is also involved in insulin secretion. We next examined the effects of 100 nM wortmannin on the insulin secretion induced by different concentrations of glucose (Fig. 2). Interestingly, the effect was glucose-dependent; the increment of insulin secretion by this agent was much smaller at lower concentrations of glucose.

Inhibition of PI3-kinase in MIN6 cells by wortmannin

We measured the activities of PI3-kinase in wortmannin-treated MIN6 cells using immunoprecipitates of MIN6 cell lysates with anti-PI3-kinase p85 antiserum. Because of irreversible binding properties of

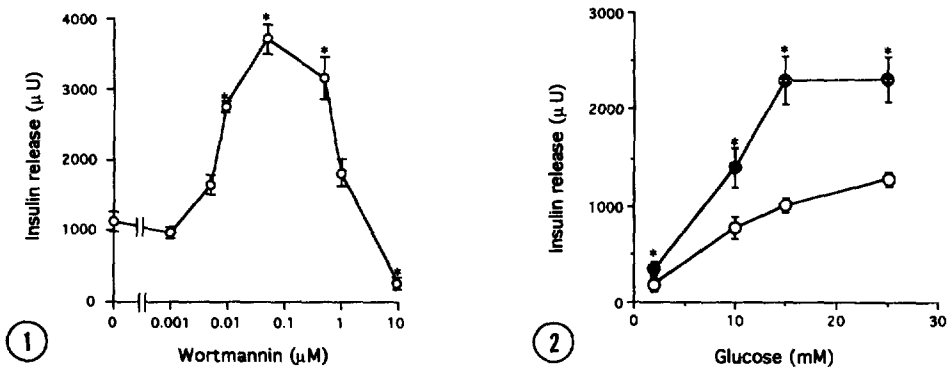


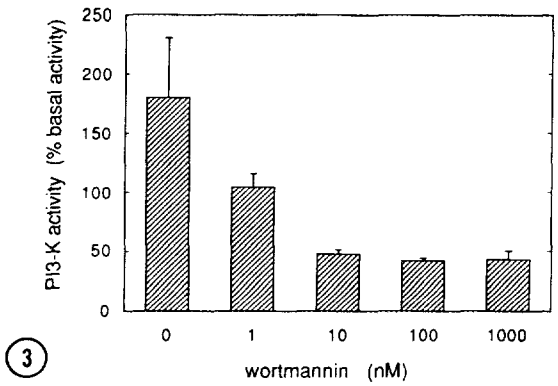
Fig. 1. Effects of wortmannin on the glucose-induced insulin secretion from pancreatic β cell line MIN6. MIN6 cells cultured in 24-well plates were preincubated with various concentrations of wortmannin at 37°C for 15 min and further incubated with 15 mM of glucose in the presence of wortmannin for 90 min. IRI secreted into the supernatants was measured. Data are the means and S.E. of triplicate wells from an experiment. Two additional experiments gave a similar result. Dimetyl sulfoxide used to dissolve wortmannin had no effects on insulin release. Significance levels: *, P< 0.05, vs control (wortmannin free).

Fig. 2. Effects of wortmannin on the insulin secretion from MIN6 cells in response to different concentrations of glucose. MIN6 cells cultured in 24-well plates were preincubated without (open circle) or with 100 nM of wortmannin (filled circle) for 15 min and further incubated with various concentrations of glucose in KRBB containing wortmannin for 90 min. IRI secreted into the supernatants was measured. Data are the means and S.E. of triplicate wells from an experiment. Two additional experiments gave a similar result. Significance levels: * p<0.05, vs control (wortmannin free).

wortmannin to PI3-kinase p110, we could measure the activity of PI3-kinase in the form of p85-p110 complex present in intact cells with this method. PI3-kinase activity was activated about two-fold by stimulation of the cells with 20 mM glucose for 15 min (Fig. 3). Wortmannin inhibited the PI3-kinase activity in intact MIN6 cells in a dose-dependent manner at concentrations ranging from 1 to 100 nM. Pretreatment of the cells with 100 nM wortmannin caused 77% inhibition of the PI3-kinase activity. The residual activity was not further suppressed by pretreating the cells even at 1 μM. This inhibitory profile is similar to that of the PI3-kinase activity in RBL-2H3 cells (9).

Binding of wortmannin to intracellular target proteins in intact MIN6 cells

We previously demonstrated direct interaction of wortmannin with the PI3-kinase p110 subunit in intact RBL-2H3 cells by immunoblot analysis using anti-wortmannin monoclonal antibody KM987. MIN6 cells



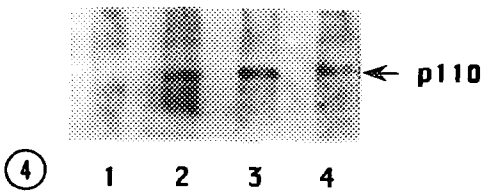


Fig. 3. Effects of wortmannin on the PI3-kinase activity in MIN6 cells. MIN6 cells cultured in 6-well plates were treated with wortmannin for 15 min then stimulated with 20 mM glucose for 15 min in the presence of wortmannin. PI3-kinase activity in the immunoprecipitates of the cell lysates with anti-PI3-kinase p85 serum was measured as described (9). PI3-kinase activities are shown as % of basal activity obtained without glucose stimulation in the absence of wortmannin. Data are the means and S.E. of triplicate wells.

Fig. 4. Immunodetection of proteins bound to wortmannin in MIN6 cells. MIN6 cells were untreated (lane 1) or pretreated with wortmannin at 30 nM (lane 2), 300 nM (lane 3), and 3 μM (lane 4) for 15 min and were then stimulated with 20 mM glucose for 15 min. Immunoprecipitates with anti-PI3-kinase p85 serum were prepared from the cells and were subjected to immunoblot analysis using anti-wortmannin antibody. The molecular mass of the protein was estimated by the following size markers: myosin heavy chain (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa).

treated with wortmannin were similarly subjected to immunoblot analysis using this antibody. However, only a 155-kDa protein band was detected in the lysates of the cells treated with wortmannin at concentrations of 0.3 and 3 μM (not shown). This protein was very likely to be MLCK from its estimated molecular mass.

Because we could not show the interaction of wortmannin with the PI3-kinase p110 subunit by this method probably due to its minor contents in the cell, the lysates of the cells treated with wortmannin were first immunoprecipitated with anti-PI3-kinase p85 serum, and were subjected to immunoblot analysis. KM987 antibody detected doublet bands corresponding to 110 kDa (Fig. 4) only in the immunoprecipitates from the cells treated with wortmannin at concentrations higher than 30 nM (Fig. 4, lanes 2-4), but not from untreated cells (Fig. 4, lane 1). Therefore, this 110 kDa protein was probably the PI3-kinase p110 subunit. The direct

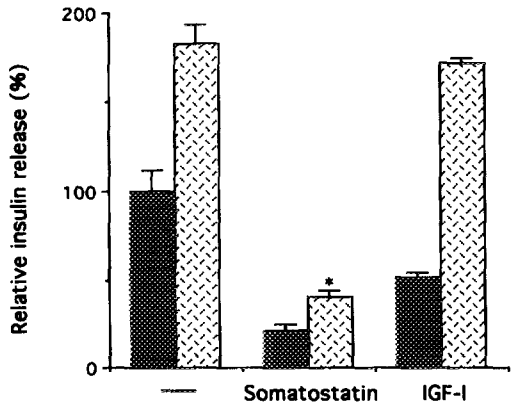


Fig. 5. Effects of wortmannin on the suppression of insulin secretion by somatostatin or IGF-I. MIN6 cells cultured in 24-well plates were preincubated in KRBB containing 5 mM glucose without (black box) or with (shaded box) 50 nM of wortmannin for 15 min followed by challenge with 10 nM of somatostatin or IGF-I. After 5 min, the medium was changed to KRBB with the same ingredients but 15 mM glucose. Supernatants were collected after incubation for 90 min, and IRI was measured. The experiment was performed in triplicates, and values (means and S.E.) are presented as % of 15 mM glucose-stimulated insulin release in the absence of wortmannin (100% correspond to 1297 μM of insulin release per well). Significance levels: * $p<0.05$, vs inhibitor free in the presence of 50 nM wortmannin.

interaction of wortmannin to PI3-kinase in intact cells was also confirmed by another method. The lysates from MIN6 cells treated with wortmannin were immunoprecipitated with KM987 antibody, and were subjected to immunoblotting. Anti-PI3-kinase p85 serum detected an 85-kDa protein band only in the immunoprecipitates from the cells treated with wortmannin at concentrations higher than 30 nM, but not from untreated cells (not shown). Taken together, these results suggest that wortmannin directly binds to the PI3-kinase p110 subunit and inhibits PI3-kinase in intact MIN6 cells, as was observed in RBL-2H3 cells (9).

Effect of wortmannin on the suppression of insulin secretion by somatostatin or IGF-I

The above experiments suggest that PI3-kinase activity mediates a suppressive action on the insulin release process. Insulin and IGF-I exert an inhibitory effect on the insulin release, which has been shown to be mediated by their binding to the IGF-I receptor on β cells (7). Recently, it has been reported that insulin and IGF-I binding to their cognate receptors results in the activation of PI3-kinase (13, 14). Insulin is capable of binding to IGF-I receptor, although at low affinity. Therefore, it seems

likely that PI3-kinase activated by insulin binding to IGF-I receptor on MIN6 cells suppresses insulin release. We investigated the effects of wortmannin on the IGF-I receptor-mediated suppression of insulin release. Ten nM of IGF-I or somatostatin decreased 15 mM glucose-induced insulin release by ~50% or ~80%, respectively (Fig. 5). Somatostatin has been shown to act at several different levels in the secretory process, including inhibition of β-cell electrical activity and ion fluxes, a decrease in the concentrations of intracellular messengers such as Ca^{2+} and cyclic AMP, and inhibition of a late stage of secretion distal to the increase in intracellular Ca^{2+} (15). When 50 nM of wortmannin was added to the culture, the suppressive effect of IGF-I was completely canceled (Fig. 5). On the other hand, the same concentration of wortmannin had only marginal effect on the somatostatin-induced suppression of insulin release. These results support the possibility that PI3-kinase is activated by insulin through IGF-I receptor and evokes suppression of insulin secretion and that wortmannin abrogates this feedback mechanism.

DISCUSSION

Insulin secretion from pancreatic β cells is positively and negatively regulated by a number of factors, such as metabolites, peptide hormones, and neurotransmitters, through various signaling pathways. In the present study, we examine the role of PI3-kinase in the regulation of insulin secretion, using wortmannin as the selective inhibitor of PI3-kinase. Wortmannin inhibits PI3-kinase activity in intact cells with an IC_{50} value of 2 nM by binding to the 110-kDa subunit (9).

The present study has demonstrated that wortmannin potentiates the glucose-induced insulin secretion in a dose-dependent manner at low concentrations (5~50 nM), but suppresses it at higher concentrations (1~10 μM). The inhibition of insulin release by 1 μM or higher wortmannin strongly suggests that MLCK is involved in the insulin translocation. Consistent with this, wortmannin was shown to bind to MLCK at concentrations higher than 0.3 μM (not shown). The present data have also shown that wortmannin bind selectively to the 110-kDa subunit of PI3-kinase and inhibit the PI3-kinase activity in MIN6 cells. The inhibition of PI3-kinase interfered with the histamine release in RBL-2H3 cells (9), but it was not the case with MIN6 cells. Wortmannin at the concentrations where it suppresses PI3-kinase activity markedly enhanced the insulin release from MIN6 cells. Potential role of PI3-kinase in the regulation of vesicular transport has been implicated on the basis of its effects on the sorting of vacuoles in yeast (16), histamine release from mast cells (9), platelet-derived growth factor receptor endocytosis (18), and cytoskeleton

changes in thrombin-stimulated platelets (19). Our present study suggests that PI3-kinase not only positively, but also negatively regulates granule release. Although its precise mechanism is not known, 3-phosphorylated inositol phospholipids, products of PI3-kinase, may be important second messengers for the regulation of insulin release. This regulation by PI3-kinase is not likely to be mediated by altering Ca^{2+} influx, because wortmannin did not affect intracellular Ca^{2+} levels (not shown).

It has been shown that insulin and IGF-I bind to IGF-I receptor which exists on the cell surface of pancreatic β cells, and reduce insulin release (7, 19). The suppressive effect of IGF-I was similarly observed in MIN6 cells (Fig. 5). It has been reported that IGF-I binding to IGF-I receptor mediates the tyrosine phosphorylation of IRS-I and increases the PI3-kinase activity (6). Because 50 nM of wortmannin completely canceled the suppressive effect of IGF-I (Fig. 5), it seems likely that this agent increased the insulin secretion by blocking the signal pathway through IGF-I receptor and PI3-kinase. Based on these data, we propose a model on the role of PI3-kinase in the regulation of insulin release as follows: insulin released from β cells binds to their own IGF-I receptor and activates PI3-kinase, and the products of PI3-kinase transmit signals to suppress insulin release. This model is further supported by the facts that PI3-kinase activity of MIN6 cells was enhanced by raising glucose concentrations of the medium (Fig. 3), and that the stimulative effect of wortmannin on insulin secretion was more prominent at higher concentrations of glucose (Fig. 2). However, it remains to be elucidated to what extent such negative feedback mechanism controls the insulin secretion *in vivo*.

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