Modulation of Tyrosine Kinase Activity Has Multiple Actions on Insulin Release from the Pancreatic β-Cell: Studies with Lavendustin A

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ABSTRACT—We investigated the role of tyrosine kinases in the regulation of insulin release from a hamster β-cell line, HIT T15, using selective tyrosine kinase inhibitors. Genistein increased the insulin release induced by glucose, but herbimycin A, tyrphostins and the erbstatin analogue failed to change the release. Lavendustin A at 0.1 nM - 1 μM caused a concave-shaped inhibition of the insulin release stimulated by 7 mM glucose. The inhibitory effect of lavendustin A was overcome by higher concentrations of glucose. Lavendustin B, the negative control analogue, had no effect on the release. Lavendustin A at a nanomolar range progressively inhibited insulin release by high K+ (50 mM)-depolarization, whereas the inhibitor did not change the insulin release by Ca2+ ionophore (A23187). On the contrary, lavendustin A at 10 nM significantly increased insulin release when glucose-induced insulin release was enhanced by either 5 μM forskolin or 162 nM 12-O-tetradecanoylphorbol 13-acetate. Lavendustin A failed to influence the Ca2+-induced insulin release from HIT cells permeabilized with streptolysin-O. These findings suggest that tyrosine kinases may play versatile roles in the control of insulin release from the pancreatic β-cell.

Keywords: Insulin release, Tyrosine kinase, Pancreatic β-cell, Lavendustin A, Tyrosine kinase inhibitor

Insulin is synthesized in and released from pancreatic β-cells distributed in the islets of Langerhans, but the regulatory mechanism of insulin release is not fully understood. The secretory activity of the β-cell is controlled by various second messenger systems including Ca2+, cAMP and products of phospholipid metabolism, and the resultant serine/threonine phosphorylation by these second messengers regulates insulin secretion (1). Phosphorylation and dephosphorylation on tyrosine residues participate in many cellular functions such as cell proliferation and differentiation, carcinogenesis, nutrient metabolism and some secretory events. Tyrosine phosphorylation of endogenous proteins has been identified from various secretory tissues when stimulated (2-5). Insulin and insulin-like growth factors, which cause tyrosine phosphorylation, have been demonstrated to control the secretory activity of exocrine and endocrine tissues to various directions (6-8). Participation of tyrosine phosphorylation/dephosphorylation in the secretory machinery has also been examined using permeabilized pancreatic acinar cells (9).

Recently, the presence of several tyrosine kinases has been identified in the pancreatic β-cell (10). Insulin and insulin-like growth factor (IGF)-1 activate tyrosine phosphorylation in the β-cell (11) and not only these hormones but prolactin and growth hormone, which activate non-receptor tyrosine kinase, have increasing effects on insulin secretion (11-13). Moreover, vanadate, which exhibits insulin-like effects via inhibition of tyrosine phosphatase, also potentiates insulin release from mouse islets (14). Glucose, the most important secretagogue of insulin release, causes tyrosine phosphorylation of endogenous proteins including the β-subunit of the insulin receptor in the β-cell (11).

In this study, we investigated the possibility that glucose-caused tyrosine phosphorylation may regulate the secretory activity of the β-cell, using various tyrosine kinase inhibitors and a hamster pancreatic β-cell line, HIT T15.

MATERIALS AND METHODS

Materials  
Genistein, lavendustin A, lavendustin B (Seikagaku
Kogyo, Tokyo), herbimycin A and tyrphostins (1, 25, B42, B44(−), B46, B48, B56) (Calbiochem, La Jolla, CA, USA) were dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO in the incubation buffer was less than 0.1010 (v/v), which did not affect insulin release and the assay in these experiments. The erbstatin analogue (Research Biochemicals, Inc., Natick, MA, USA) was dissolved in 0.1 M NaOH. The phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) and the Ca2+ ionophore A23187 (Sigma Chemical Co., St. Louis, MO, USA) were also dissolved in DMSO at 324 pM and 10 mM respectively. Forskolin (Wako, Tokyo) was dissolved at 10 mM in ethanol. BSA (Fraction V) was purchased from Wako. Streptolysin-O (STLO) and insulin radioimmunoassay kit were from Eiken Chemical (Tokyo). All other substances were of the purest grade available.

Cell culture

HIT T15 β-cells (passage numbers 63–90), kindly donated by Prof. S. Seino (Chiba University School of Medicine, Chiba), were cultured at 37°C in a humidified atmosphere of 95% air / 5% CO2 in Ham's F-12K medium (ICN Biomedicals, Costa Mesa, CA, USA) with kanamycin (30 pg/ml) and 10010 fetal calf serum. This cell line retains good secretory responses to agents including glucose, although the dose-response curve to the hexose is shifted to the left (15). The cells were passaged weekly and harvested by using trypsin/EDTA and culture medium was replaced every other day.

Insulin secretion

HIT cells were seeded at a density of 3.5–5 x 105 cells per well in 24-well tissue culture plates 2 days prior to each experiment. On the day of the experiment, cells were preincubated at 37°C for 2 hr in 1 ml of Hepes-Krebs buffer containing: 119 mM NaCl, 4.75 mM KCl, 5 mM NaHCO3, 2.54 mM CaCl2, 1.2 mM MgSO4 and 20 mM Hepes (pH 7.4 with NaOH), with 5 mg/ml BSA. Glucose-free preincubation is required to reveal secretory sensitivity to glucose of HIT cells (15). After preincubation, the cells were further incubated for 1 hr in 0.5 ml of Hepes-Krebs buffer containing 5 mg/ml BSA with or without the various reagents described. When K+ concentration was heightened, Na+ was reduced iso-osmotically. At the end of the incubation, an aliquot was collected, centrifuged briefly to sediment any detached cells and stored at −20°C until assayed. Insulin release was measured by radioimmunoassay using bovine insulin as the standard. Insulin release from HIT cells in these conditions was approximately 0.5–5.2% of the insulin content. In some experiments, glucose stimulation was carried out at different concentrations since the secretory response of the β-cell line to glucose changes depending on the passage. Because lavendustin A failed to change the release when the β-cell was maximally stimulated with glucose (see Fig. 2), we checked the glucose sensitivity of HIT cells and chose a glucose concentration that gave a 50–70% increase of the maximal stimulation by glucose (5–10 mM).

Cell permeabilization

HIT cells were permeabilized with STLO as previously described (16). For these experiments, we used glutamate buffer containing: 100 mM K-glutamate, 42 mM Na-glutamate, 16 mM Hepes (pH 7.0), 3 mM MgATP, 1 mM EGTA and 5 mg/ml BSA. CaCl2 was added to the glutamate buffer to give an arbitrary concentration of free Ca2+. After preincubation for 1 hr at 37°C in 1 ml of Hepes-Krebs buffer containing 5 mg/ml BSA, the HIT cells were washed twice with 0.7 ml of the glutamate buffer with 100 nM Ca2+, and further incubated for 45 min in 0.5 ml of the glutamate buffer plus 500 U/ml STLO at either 100 nM or 10 μM Ca2+. The preliminary experiment suggested that this concentration of STLO evokes the maximal secretory response of the insulin to 10 μM Ca2+. At the end of the 45 min incubation, the medium was collected for the insulin assay as described above.

Statistical analyses

Statistical significance of the data was assessed by the unpaired Student's t-test.

RESULTS

Effects of various tyrosine kinase inhibitors on glucose-induced insulin secretion from HIT cells

Effects of different types of tyrosine kinase inhibitors on glucose-induced insulin release are summarized in Table 1. Neither herbimycin A, the erbstatin analogue, nor tyrphostins (1, 25, B42, B44(−), B46, B48 and B56) changed the insulin release induced by glucose, whereas 100 pM genistein increased and 10 nM lavendustin A decreased the insulin release from HIT cells.

Effects of lavendustin A on glucose-induced insulin release from HIT cells

Effects of lavendustin A (0.1 nM–1 μM) on insulin release stimulated by 7 mM glucose are shown in Fig. 1, where a concave-shaped decrease in insulin release by the inhibitor was demonstrated. A maximum inhibition of 54% was observed at 10 nM, but the value returned to the control level at 1 μM lavendustin A. In contrast, lavendustin B, a negative analogue of lavendustin A, within a range between 1 nM and 1 μM did not change the glucose-induced insulin release (data not shown).
Table 1. Effects of tyrosine kinase inhibitors on glucose-stimulated insulin release from HIT cells

| Glucose (mM) | Inhibitors            | Insulin release (%) |
|--------------|-----------------------|---------------------|
| none         |                       | 100                 |
| 10           | crbstatin analogue (100 μM) | 97.0 ± 5.7 (6)      |
| 7            | herbimycin A (1 μg/ml) | 101.8 ± 7.7 (6)     |
| 10           | genistein (100 μM)    | 207.1 ± 5.4* (4)    |
| 10           | tyrphostin 1 (100 μM) | 90.0 ± 3.0 (5)      |
| 10           | tyrphostin 25 (100 μM)| 127.5 ± 19.2 (5)    |
| 10           | tyrphostin B42 (100 μM)| 115.0 ± 2.9 (5)     |
| 10           | tyrphostin B44 (−) (100 μM)| 108.5 ± 9.1 (5)   |
| 10           | tyrphostin B46 (100 μM)| 141.5 ± 18.9 (5)    |
| 10           | tyrphostin B48 (100 μM)| 107.0 ± 7.7 (5)     |
| 10           | tyrphostin B56 (100 μM)| 149.0 ± 19.2 (5)    |
| 7            | lavendustin A (10 nM) | 46.0 ± 6.5* (10)    |

After a 2-hr incubation without glucose, HIT cells (3.5–5 × 10⁵ cells per well) were incubated for 1 hr with stimulatory concentrations of glucose and various types of tyrosine kinase inhibitors. Since the secretory sensitivity of this cell line to glucose was passage-dependent, HIT cells were incubated with glucose at a concentration that gives 50–70% stimulation of the maximal release. Insulin released during the incubation was assayed by radioimmunoassay. Data are expressed as a percentage of the release without inhibitors in each experiment and given as mean ± S.E. for the number of observations shown in parentheses. *P < 0.01 vs control value (7 or 10 mM glucose) without inhibitors.

Figure 2 shows effects of lavendustin A on insulin release by different concentrations of glucose. This inhibitor did not influence insulin secretion in the absence of glucose. When the HIT β-cell was stimulated by increasing concentrations of glucose from 3 to 20 mM, lavendustin A at 10 nM inhibited insulin release in the middle of the concentration range, whereas the inhibition disappeared under maximal stimulation by glucose (>10 mM).

**Effects of lavendustin A on insulin release under various stimuli**

Lavendustin A also inhibited insulin release from HIT cells induced by 50 mM K⁺ (Fig. 3). Elevation of the

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Fig. 1. Effects of lavendustin A on glucose-induced insulin release from HIT cells. HIT cells were preincubated in glucose-free, BSA (5 mg/ml)-containing Heps-Krebs buffer for 2 hr, followed by incubation in the same buffer containing glucose and various concentrations of lavendustin A (0.1 nM–1 μM) for 1 hr. Aliquots of the incubation buffer were centrifuged and assayed for insulin. Each symbol is the mean ± S.E. for 6–12 observations. *P < 0.05, compared with the value without lavendustin A.

Fig. 2. Effect of lavendustin A on insulin release evoked by increasing concentrations of glucose. HIT cells (3.5–5 × 10⁵ cells/well) were incubated with 0–20 mM glucose in the presence (●) or absence (○) of 10 nM lavendustin A. Insulin released into the media after 1 hr was assayed by radioimmunoassay. The results represent the mean ± S.E. of 6–10 observations from 4 separate experiments after converting the data to % of control value (0 mM glucose). *P < 0.05, compared with the value under the parallel condition without the inhibitor.

Fig. 3. Dose-dependent inhibition by lavendustin A of high K⁺-induced insulin release from HIT cells. HIT cells preincubated in a glucose-free condition for 2 hr were incubated for 45 min in BSA-containing Heps-Krebs buffer supplemented with 50 mM K⁺ and increasing concentrations of lavendustin A. Values are expressed as the mean ± S.E. for 6–12 observations. *P < 0.05, compared with the control in the absence of lavendustin A.
extracellular K+ level from 5.5 to 50 mM caused a 15-fold increase in insulin release (0.25±0.02 for control vs 3.65±0.28 ng/well/60 min for 50 mM K+, n=4-6).

Dose-dependent inhibition by lavendustin A at 0.1 nM was observed when insulin release was evoked by high K+ depolarization.

When insulin release was increased by the Ca2+ ionophore A23187 (0.38±0.04 for control vs 0.68±0.04 ng/well/30 min for 2 μM A23187, n=4–6), addition of 10 nM lavendustin A did not change the release from HIT cells (0.66±0.07 ng/well/30 min, n=6).

Table 2 demonstrates the effects of lavendustin A on insulin release enhanced by TPA and forskolin.

| Glucose (mM) | Additions | Insulin release (%) | Lavendustin A (nM) |
|--------------|-----------|---------------------|-------------------|
| 5            | none      | 100                 | 0                 |
| 5            | TPA (162 nM) | 216.3±6.7            | 266.3±5.8*        |
| 7            | none      | 100                 | 0                 |
| 7            | forskolin (5 μM) | 435.0±35.6          | 554.4±28.8*       |

HIT cells were incubated with or without 10 nM lavendustin A in glucose-containing Hepes-Krebs buffer in the presence or absence of TPA or forskolin as indicated. Since the secretory response of the β-cell line to glucose changed depending on the passage, glucose stimulation was carried out at different concentrations that gave 50–70% increase of the maximal stimulation by glucose. Data are expressed as percentage of the values of insulin release by glucose at 5 mM (for TPA) and 7 mM (for forskolin). Values are the means±S.E. for 4–6 observations. *Significantly greater than the control without lavendustin A under a parallel condition (P<0.05).

Lavendustin A, derived from Streptomyces griseola-vendus, potently inhibits epidermal growth factor (EGF) receptor tyrosine kinase, via competing with both ATP and the peptide substrates (21), while this substance has little effect on phosphatidylinositol kinase and no effect on protein kinases A and C (22). Our secretion experiments using lavendustin A demonstrated that this inhibitor, at concentrations around its IC50 value (11.5 nM) on EGF receptor tyrosine kinase (22), has different effects depending on the stimulus for insulin release. It is likely that β-cell tyrosine kinase(s) may play multiple roles in the signal transduction system of insulin secretion. Lavendustin A decreased insulin release by high K+ which depolarizes plasma membranes and opens voltage-dependent Ca2+ channels. High K+ depolarization also causes an increase in tyrosine phosphorylation of endogenous proteins in the β-cell (11). Therefore, it could be possible that tyrosine phosphorylation is involved in the Ca2+-signaling for insulin release in the β-cell.

DISCUSSION

Pharmacological agents that modulate the activities of protein kinases/phosphatases are very useful for studying possible roles of these enzymes in physiological/pathological phenomena. Tyrosine kinase inhibitors have also been applied to secretion studies to elucidate the involvement of protein phosphorylation on tyrosine residues in the secretory machinery of various tissues (3, 6, 17, 18). Here, we examined the effects of different types of tyrosine kinase inhibitors on insulin release and found that these inhibitors have variable effects on the secretory activity. In the previous reports, two structurally dissimilar inhibitors of tyrosine kinase, genistein and herbimycin A, were found to increase glucose-induced insulin release from neonatal rat islets (12) and from mouse islets (19). Indeed, genistein increased glucose-induced insulin release from HIT cells in the present study. It must be born in mind that genistein has been reported to increase cAMP content presumably via inhibition of cyclic nucleotide phosphodiesterase in a β-cell line, MIN6 (20). Moreover, Jonas et al. (19) extensively investigated the mechanisms by which genistein augments insulin release from mouse islets and suggested this compound may act on an unknown mechanism(s) that is (are) not related to inhibition of tyrosine kinases. On the contrary to these reports, herbimycin A did not change the insulin release from the HIT β-cell in our hands. Inconsistent results between other experiments and ours may be due to differences in the cells used.

Lack of effect of lavendustin A on insulin release from permeabilized HIT cells

In STLO-permeabilized HIT cells, elevation of free Ca2+ from 100 nM to 10 μM caused an approximately 2.5-fold increase in insulin release. Lavendustin A (10 nM) failed to influence the insulin release from HIT cells permeabilized with STLO in the presence or absence of a stimulatory concentration of Ca2+ (at 100 nM Ca2+: 0.81±0.04 for control vs 0.78±0.03 ng/well/45 min with 10 nM lavendustin A, n=6, NS; at 10 μM Ca2+: 2.16±0.20 for control vs 2.11±0.28 ng/well/45 min with 10 nM lavendustin A, n=6, NS).

Table 2. Effects of lavendustin A on insulin release enhanced by TPA and forskolin

HIT cells were incubated with or without 10 nM lavendustin A in glucose-containing Hepes-Krebs buffer in the presence or absence of TPA or forskolin as indicated. Since the secretory response of the β-cell line to glucose changed depending on the passage, glucose stimulation was carried out at different concentrations that gave 50–70% increase of the maximal stimulation by glucose. Data are expressed as percentage of the values of insulin release by glucose at 5 mM (for TPA) and 7 mM (for forskolin). Values are the means±S.E. for 4–6 observations. *Significantly greater than the control without lavendustin A under a parallel condition (P<0.05).
the inhibitory effect of lavendustin A disappeared when insulin release from STLO-permeabilized HIT cells was directly stimulated by Ca\textsuperscript{2+}, inhibition by lavendustin A may result from modulation of Ca\textsuperscript{2+} fluxes across plasma membranes through Ca\textsuperscript{2+} channels/pumps as suggested for pancreatic acinar cells (23). This idea was further supported by the present finding that lavendustin A failed to change insulin release evoked by the Ca\textsuperscript{2+} ionophore A23187 and also supported by the recent report that activation of Janus (JAK) tyrosine kinase by growth hormone and prolactin increases Ca\textsuperscript{2+}-influx through voltage-dependent Ca\textsuperscript{2+} channels in another insulinoma cell line, INS-1 (24). Growth hormone failed to influence glucose-induced insulin release, but stimulated DNA synthesis in the insulinoma cells, which was abolished by lavendustin A (24). An alternative explanation for lack of effects of lavendustin A in STLO-treated cells is that tyrosine kinases and/or their substrates could be simply lost under that experimental condition for membrane permeabilization since STLO forms rather large pores in plasma membranes (25).

Unexpectedly, lavendustin A at 10 nM, which inhibited glucose- or high K\textsuperscript{+}-evoked release, increased insulin release by co-incubation with glucose and either forskolin or TPA. One simple interpretation is that activation of tyrosine kinase(s) may regulate insulin release by activation of protein kinases A and C to an opposite direction to Ca\textsuperscript{2+}-dependent release. Negative control by tyrosine phosphorylation against protein kinase A- or C-dependent secretion has been also suggested in other tissues (7, 8). Glucose stimulates insulin release via activation of several protein kinases by Ca\textsuperscript{2+}, cAMP and diacylglycerol (26). Since H-7, an inhibitor of protein kinases A and C (27), decreases insulin release from rat islets at a high (16.7 mM) concentration of glucose, these kinases appear to be involved in insulin release with high glucose (28). That could be the reason why lavendustin A caused concave-shaped inhibition of insulin release within a limited concentration range of glucose. It is also possible that different types of tyrosine kinase inhibitors have variable effects on insulin release because the inhibitors exhibit distinct specificities against various tyrosine kinases as suggested for lavendustin A (29). The responsible substrates for the control of insulin secretion remain to be identified in this study. P29, a synaptophysin-like vesicle protein, could be one of the candidates since the protein is distributed in neuronal/endocrine cells and phosphorylated by endogenous tyrosine kinase activity (30).

These findings and previous reports suggest us that tyrosine kinases may participate in signal cross-talk of the transduction systems in the pancreatic ß-cell.

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