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Overexpressed Syntaxin 1A/HPC-1 Inhibits Insulin Secretion Via a Regulated Pathway, but Does Not Influence Glucose Metabolism and Intracellular Ca^{2+} in Insulinoma Cell Line βTC3 Cells

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We have previously established a stable βTC3 cell line that overexpresses syntaxin 1A, designated βTC-hpc1 cells, in which glucose-stimulated insulin release was decreased. Using βTC-hpc1 cells, we aimed to determine whether syntaxin 1A functions in the regulatory or constitutive pathway of insulin release. We therefore examined the secretion of phorbol-12-myristate-13-acetate (TPA)-stimulated newly synthesized proinsulin/insulin and total immunoreactive insulin. βTC3 and βTC-hpc1 cells were simultaneously pulse-labeled with ^{3}H-leucine for 30 min in 11 mM glucose and chased for 1 h in one of a number of different concentrations of TPA in 11 mM glucose. Total immunoreactive insulin release (IRI) by both cell types during the chase period was markedly increased by the addition of TPA in a dose-dependent manner; however, the IRI from βTC-hpc1 cells was lower than that from βTC3 cells. The secretion of newly synthesized proinsulin/insulin from both cell types, which in βTC3 cells is thought to occur via a constitutive pathway, was in the same range under any condition. Thus, the evidence indicates that syntaxin 1A preferentially functions in the regulated insulin release pathway in βTC3 cells. In order to clarify the effect of overexpressed syntaxin 1A on glucose metabolism and intracellular Ca_{2+} we analyzed the glucose transport system, glucose phosphorylation activity, and cytosolic concentration of free Ca^{2+} ($[Ca^{2+}]_i$). 2-Deoxy-glucose uptake and the content of GLUT1 protein in the plasma membrane fractions of βTC-hpc1 cells were not different from those of βTC3 cells. Radiometric assays of glucose phosphorylation activity showed that there were no differences in hexokinase activity and glucokinase activity between βTC3 and βTC-hpc1 cells. $[Ca^{2+}]_i$ measured by using fura 2 demonstrated that there was no difference in $[Ca^{2+}]_i$ between βTC3 and βTC-hpc 1 cells under glucose-stimulated conditions. The present experiments indicate that syntaxin 1A plays a central role in a late step of the regulatory insulin release pathway without a change in glucose metabolism and $[Ca^{2+}]_i$ in βTC3 cells.

Regulated secretory cells have two pathways for the release of a secretory product, the so-called regulated and constitutive pathways (1). In the regulated pathway of pancreatic β cells, insulin release arises in response to a stimulus such as glucose, forskolin, and TPA (2-4), and in the constitutive pathway insulin is packaged into vesicles, which are unlike secretory granules, that are destined for rapid release in a non-regulated fashion. In mouse βTC3 cells, the preferential release of newly synthesized proinsulin/insulin has been shown to be a reflection of its release via a constitutive pathway, and the regulatory insulin release from stored secretory granules has actually been measured by total immunoreactive insulin release (IRI)(5). Thus, βTC3 cells are a suitable cell line in which to study the regulated or constitutive pathway of insulin release. Therefore, using syntaxin 1A-overexpressing βTC-hpc1 cells, we examined whether syntaxin 1A functions in either the regulatory or constitutive pathway of insulin release by measuring newly synthesized proinsulin/insulin release and IRI.

There is a considerable body of evidence to indicate

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Abbreviations used: SNAP, synapse associated protein; SNARE, SNAP receptor; SNAP-25, synaptosomal-associated protein of 25 kDa; GLUT, facilitated glucose transporter; PAGE, polyacrylamide gel electrophoresis.

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that syntaxin 1A plays an important role in exocytosis. Evoked neurotransmitter release is abolished in Drosophila mutants lacking syntaxin 1A (6). Broadie *et al.* have also demonstrated, using Drosophila strains lacking synaptobrevin or syntaxin, that syntaxin is required for the actual fusion event between vesicle and plasma membranes (7). Experiments using specific antibodies against syntaxin 1A showed that the antibody treatment inhibited calcium-dependent insulin secretion (8) and catecholamine secretion (9). On the other hand, in pancreatic β cells, since glucose metabolism and intracellular Ca^{2+} are closely associated with the regulation of insulin secretion (10-15), alteration of those caused by syntaxin 1A overexpression in βTC-hpc1 cells may affect insulin release. In the present study, in order to define the relationship between glucose metabolism, $[Ca^{2+}]_i$, and overexpression of syntaxin 1A, we analyzed the glucose transport system, hexokinase and glucokinase activity, and cytosolic concentration of free Ca^{2+} in βTC-hpc 1 cells.

MATERIALS AND METHODS

Materials. Dulbecco's Modified Eagle's Medium (DMEM), RPMI 1640, and fetal bovine serum were obtained from GIBCO BRL. Leucine-deficient RPMI 1640 medium was made using a selected amine kit from GIBCO BRL. L-[4,5-^{3}H] Leucine (145 Ci/mmol), [U-^{14}C] glucose and 2-[1-^{14}C] deoxy -D-glucose were obtained from Amersham Corp. A DC-protein assay kit was from Bio-Rad. Nitrocellulose membranes were purchased from Schleicher and Schuell. Phorbol-12-myristate-13-acetate (TPA) was obtained from Sigma. All other reagents used were of the highest quality commercially available.

Cell culture. A βTC-hpc1 cell line stably overexpressing rat syntaxin 1A/HPC-1 was established as described previously (16). This cell line, and the parent βTC3 cell line (kindly provided by Dr. D. Hanahan, UCSF, San Francisco) were cultured and maintained in DMEM. For pusle-chase experiments, the cells were incubated in RPMI 1640 medium [10% (v/v) dialyzed fetal bovine serum] containing the indicated concentrations of TPA in 11 mM glucose.

Pulse/chase labeling of cells. βTC-hpc1 and βTC3 cells were pulse-labeled for 30 min at 37 °C in 1ml leucine-deficient RPMI 1640 [10% (v/v) dialyzed fetal bovine serum] containing 11 mM glucose and 1.0 mCi L-[4,5-^{3}H] leucine. This concentration of glucose was used so as to stimulate proinsulin biosynthesis (5) and hence cause maximum [^{3}H] leucine incorporation into newly synthesized endogenous proinsulin. Pulse-labeling of cells was stopped by washing twice in complete RPMI-1640 medium, and then chased in complete medium containing either 0, 0.5, 1, 5, or 50 nM TPA for 1 h. At the end of each chase period an aliquot of the chase medium was removed and centrifuged in a 1.5 ml-microfuge at 15,000 rpm for 5 min (to pellet any cell debris). The pellet was discarded and the supernatant stored at −20 °C. Cells were washed twice in ice-cold phosphate buffered saline (PBS), harvested, and pelleted by microcentrifuge. Labeled cell pellets were extracted by sonication and stored at −20 °C pending analysis.

Analysis of the chase medium. The total IRI in the aliquots of chase medium was assayed by radioimmunoassay using a ^{125}I-rat insulin assay kit (Amersham Corp.). ^{3}H-labeled proinsulin/insulin from the cells and the chase media were immunoprecipitated with guinea pig anti-insulin antiserum (DAKO), as described previously (5). ^{3}H-Labeled hormone secretion was expressed as the fractional secretion rate of newly synthesized proinsulin/insulin, which was derived as follows: total [^{3}H] proinsulin and insulin secreted/final [^{3}H] proinsulin and insulin plus total [^{3}H] proinsulin and insulin secreted.

Assay of 2-deoxyglucose uptake. The assay was essentially performed as described previously (5). Briefly, cells were incubated for 4 h in Hank's buffer, after which 0.5 μCi 2-[^{14}C] deoxy-D-glucose was added to the medium containing 0.1 mM unlabeled 2-deoxy-D-glucose. Nonspecific uptake was assessed by assay in the presence of 50 μg/ml cytochalasin-B. After 5 min at room temperature, cells were washed with ice-cold PBS, solubilized by the addition of 0.2 M NaOH, and the radioactivity was counted using a liquid scintillation counter.

Subcellular fractionation and immunoblot analysis. The plasma membrane fractions were obtained by the sucrose gradient method, as described previously (16). They were assayed to determine their protein content using the Bio-Rad Protein Assay kit (Bio-Rad), subjected to 10% SDS-PAGE, and then transferred to nitrocellulose membranes. Immunoblot analysis was performed essentially as described previously (16). After membranes were reacted with an anti-GLUT1 antibody (a gift from Dr. K. Takata, University of Gunma, Maebashi, Japan), they were incubated with a horseradish peroxidase-conjugated anti-rabbit IgG antibody. Immunoreactivities were determined using the chemiluminescence reaction (DuPon, NEN). The relative amounts of the detected bands were quantified by laser-scanning densitometry (Image Master Ver1.20, Pharmacia Biotech Corp.).

Measurement of glucose phosphorylation. Hexokinase and glucokinase activity were measured in βTC3 and βTC-hpc1 cell extracts, as described previously (17). Briefly, cells were disrupted by sonication, and centrifuged at 100,000 g for 1 h, and the resulting supernatants were used for assay. Hexokinase activity was measured using 0.5 mM glucose as a substrate and by measuring the conversion of [U-^{14}C] glucose to [U-^{14}C] glucose-6-phosphate. Glucokinase activity was determined from assays performed with a substrate of 20 mM glucose, in the absence or presence of 10 mM unlabeled glucose-6-phosphate, a potent inhibitor of hexokinase (18). Data are expressed as the fold increase of kinase activity obtained for βTC3 cells in each experiment.

Measurement of $[Ca^{2+}]_i$. For single cell measurement of $[Ca^{2+}]_i$ in a microscopic system, βTC3 and βTC-hpc1 cells were cultured on a glass cover-slips in RPMI1640 containing 11 mM glucose for 24 h and loaded with a membrane permeable form of fura-2 (fura-2/AM, Dojindo, Kumamoto) at a concentration of 2 μM for 30 min at 37°C in the culture medium. Also cremophore (0.2%, Sigma) was added. Cells were then rinsed in normal saline-Hepes buffer: NaCl 160 mM; KCl 5 mM; $CaCl_2$ 2.5 mM; $MgCl_2$ 1 mM; glucose 6 mM; Hepes 10 mM (pH7.4). Fluorescence measurements were performed either in normal saline-Hepes or high K^+-solution (KCl 50 mM; NaCl 115 mM). The bath solution was perfused at 1 ml/min by a peristaltic pump (P-3, Pharmacia). Fluorescence images excited by light from a xenon lamp at wavelength of 340 nm (F_{340}) and of 380 nm (F_{380}) alternatively, were obtained at every 10 s with an image processor (Argas-50, Hamamatsu Photonics, Hamamatsu). Data were processed to calculate the ratio, F_{340}/F_{380}. From this ratio, intracellular free Ca^{2+} concentration was estimated using a calibration curve according to the equation derived by Grynkiewicz, G., et al. (19).

Statistical analysis. The statistical methods were Student's *t* test or ANOVA in multiple comparisons. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

Function of Syntaxin 1A in the Regulated Insulin Release Pathway

Stable transfectant cells (βTC-hpc1), which overexpressed rat syntaxin 1A protein about 10-fold relative to that of the parent βTC3 cells, were cloned as described previously (16). To examine the function of syn-

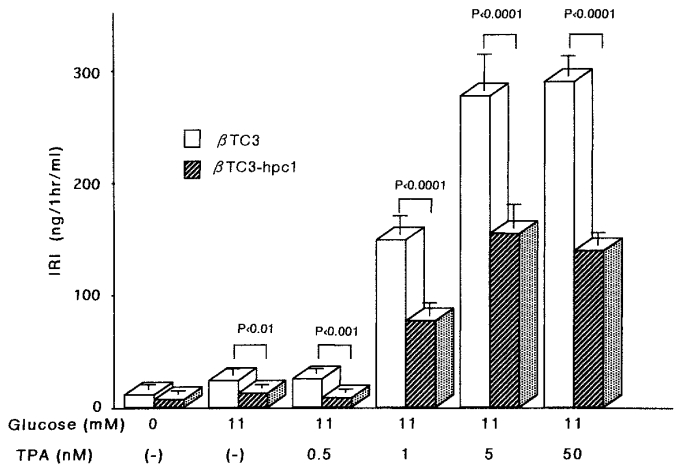

FIG. 1. Total immunoreactive insulin secretion (IRI) during a 60-min chase incubation by βTC-hpc1 and βTC 3 cells. After cells were pulse-labeled with ^{3}H-leucine for 30 min, they were chase-incubated with 0. 0.5, 1, 5, or 50 nM TPA under 11 mM glucose. The supernatants were collected and assayed for IRI.

taxin 1A in the regulated or constitutive insulin release pathway, we measured both total IRI and ^{3}H-labeled (newly synthesized) proinsulin/insulin release by performing pulse-chase experiments. Although most of the newly synthesized proinsulin / insulin from isolated rat islets is released via a regulated pathway (2, 20), it has been shown that βTC3 cells release most of IRI via a regulated pathway, and that newly synthesized proinsulin/insulin is secreted via a constitutive pathway (5). βTC-hpc1 and βTC3 cells were pulse-labeled with ^{3}H-leucine for 30 min in 11mM glucose, after which they were chased for 1 h under conditions of either 0, 0.5, 1, 5, or 50 nM TPA in 11 mM glucose. Since TPA markedly induced IRI via a regulatory pathway over just a 1 h incubation period (5, 16), we chose this agent as a secretogogue in the study described here. Total IRI secreted into the medium over the 1 h chase period was measured by insulin radioimmunoassay. As shown in Fig. 1, TPA stimulated markedly IRI from both cell types in a dose-dependent manner, but that by βTC-hpc1 cells was about half of that of βTC3 cells. In contrast, no difference in newly synthesized proinsulin/insulin secretion was observed over the 1h chase period under any condition, between βTC3 and βTC-hpc1 cells, even in condition of high concentration of TPA (Fig. 2). Thus, the results demonstrate that the overexpressed syntaxin 1A in βTC3 cells caused a reduction in IRI via a regulatory pathway, but did not affect the newly synthesized proinsulin/insulin secretion that occurred via a constitutive pathway.

From this result, it is conceivable that the expression of syntaxin 1A is associated with the regulatory rather than constitutive pathway in the cell. However, Bittner *et al.* showed that syntaxin 1A caused the reduction in the constitutive release of marker proteins from COS-1 cells that posses only the constitutive but not regulated pathway (21). Thus, it appears that not only syntaxin 1A expression, but also its expression in a suitable cell type, might be required for syntaxin 1A to preferentially act in the regulated pathway.

Relationship between Overexpressed Syntaxin 1A and Glucose Metabolism

It seemed possible that reduction of insulin release by syntaxin 1A overexpression might be connected with an alteration of glucose metabolism in βTC-hpc1 cells. Because the expression of glucose transporter (22-25) and glucose phosphorylating enzymes (15, 26-28) is known to be closely related to the regulation of insulin secretion and biosynthesis in pancreatic β cells, we measured the glucose transport system and hexokinase and glucokinase activity in βTC-hpc1 cells. In order to examine alterations in the glucose transport system of βTC-hpc1 cells, we measured the ^{14}C-2-deoxyglucose uptake and glucose transporter content in the plasma membrane fractions. As shown in Fig. 3, there was no difference in ^{14}C-2 deoxyglucose uptake between βTC3 and βTC-hpc1 cells. Since βTC3 cells predominantly expressed the GLUT 1 (17), we measured the GLUT 1 content in the plasma membranes using immunoblot analysis. GLUT 1 content tended to very slightly decrease (91 ± 11%: n=3) in the plasma membranes prepared from βTC-hpc1 cells, compared with βTC3 cells, but the difference was not significant. Although this is a negative result, this observation is interesting since it supports the evidence that syntaxin 1A preferentially functions in the regulatory pathway. Because the intracellular GLUT 1 protein trafficking that targets plasma membranes is known to be little affected by extracellular signals such as insulin (29-31), GLUT 1 movement within the cell is thought to be mediated via a constitu-

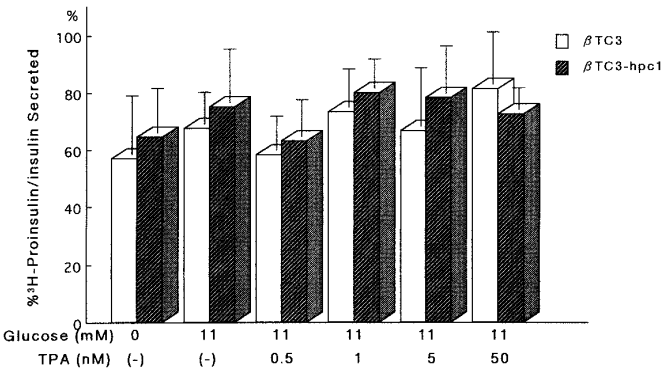

FIG. 2. Secretion of labeled proinsulin/insulin during a 60 min-chase incubation of ^{3}H-labeled βTC-hpc1 and βTC3 cells. Cells were labeled with ^{3}H-leucine for 30 min in 11 mM glucose, and then they were chased for 60 min with a various concentrations of TPA. Proinsulin/insulin from cells and media were immunoprecipitated with guinea pig anti-insulin antibody, and then fractional secretion of ^{3}H-labeled proinsulin/insulin was calculated as described under Materials and Methods.

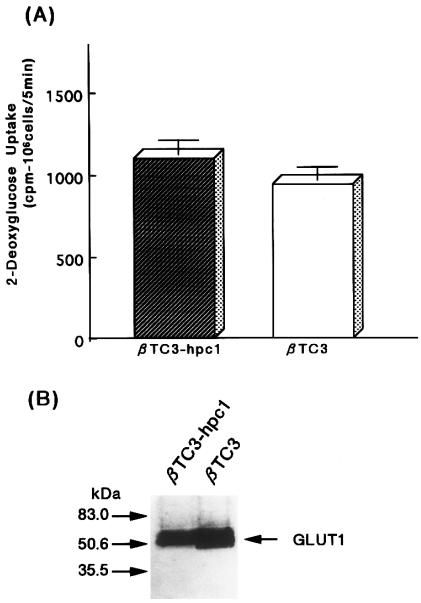

FIG. 3. Glucose transport system in βTC-hpc1 and βTC 3 cells. (A) 2-deoxy-D-glucose uptake. After cells were cultured in DMEM culture medium (22 mM glucose), they were placed in Hank's buffer for 4h. They were then incubated with 0.5 μ Ci 2-[1-^{14}C]deoxy-D-glucose under 0.1 mM unlabeled 2-deoxy-D-glucose for 5 min at room temperature. Values (n=3) represent the difference between the total [^{14}C]2-deoxyglucose uptake and the radioactivity taken up in the presence of 50 μ g/ml cytochalasin-B (nonspecific uptake). (B) Immunoblot analysis of GLUT1 glucose transporter proteins in the plasma membrane fraction. After the plasma membranes were prepared from βTC-hpc1 and βTC 3 cells by sucrose gradient method, the same amounts of protein were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-GLUT1 antiserum. Typical experiment was shown as representative of three separate experiments. The relative amounts of detected bands were quantitated by laser-scanning densitometry.

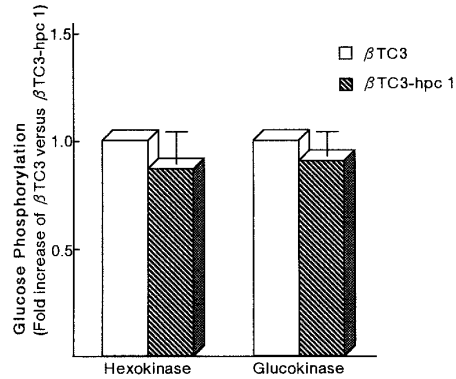

FIG. 4. Glucose phosphorylating activities in βTC-hpc1 and βTC 3 cells. Cells were maintained in DMEM culture medium as described under Materials and Methods. Cytosolic fractions were prepared from both cells, and then enzymatic assays were performed radiometrically as described under Materials and Methods. Data are expressed as the relative fold of values obtained for βTC 3 cells.

tive pathway. Thus, the data demonstrate that syntaxin 1A had no affect on either the glucose uptake rate or the glucose transporter protein traffic via a constitutive membrane fusion system.

Several laboratories have reported recently that the expression and activity of glucokinase or hexokinase regulates directly the secretion of insulin (28, 29). Therefore it is also conceivable that the decreased insulin secretion observed in βTC-hpc1 cells may be a result of the inhibition of hexokinase or glucokinase activity by the overexpression of syntaxin 1A. However, the activity of hexokinase and glucokinase in βTC-hpc1 cells was no different from that in βTC 3 cells under glucose-stimulated condition (Fig. 4). Thus, our data excluded the possibility that the function of syntaxin 1A is coupled to glucose transport and its phosphorylation.

No Effect of Overexpressed Syntaxin 1A on Intracellular Ca^{2+}

The cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$), in addition to glucose metabolism, is one of the most important regulators of insulin secretion from pancreatic β cells. On the other hand, syntaxin 1 is tightly associated with calcium channels that are involved in neurotransmitter release (32-34). Therefore it is speculated that overexpressed syntaxin 1A influences $[Ca^{2+}]_i$ in βTC 3 cells, resulting in inhibition of insulin secretion. However, as shown in Table 1, basal $[Ca^{2+}]_i$ in βTC 3 cells was not different from that of βTC-hpc1 cells under glucose-stimulated conditions. Since the Ca^{2+} influx pattern in βTC 3 cells represents the predominant expression of the L-type Ca^{2+} channel (data not shown), in agreement with other studies of pancreatic β cells (13,35, 36), 50 mM KCl was loaded into cells in order to open the L-type Ca^{2+} channel, thereby enabling the channel activity to be measured. Table 1 demonstrates that there was no difference in $[Ca^{2+}]_i$ between KCl-loaded βTC 3 and βTC-hpc1 cells. Thus, the overexpressed syntaxin 1A in βTC 3 cells did not regulate $[Ca^{2+}]_i$, which was probably mediated by Ca^{2+} carriers such as Ca^{2+} channels and Ca^{2+}-ATPase. Our results also agree with the report by Bezyprozvanny *et al.* (37), demonstrating that syntaxin 1A decreased the avail-

TABLE 1

Cytosolic Concentrations of Free Ca^{2+}[Ca^{2+}]i in βTC3 and βTC3-hpc1 Cells

	[Ca^{2+}]i (nmol/liter)	
	βTC3	βTC3-hpc1
Basal	28 ± 0.3 (28)	28 ± 0.7 (27)
KCl-loaded	103 ± 2.5 (28)	110 ± 2.4 (28)

Note. Cells were incubated in RPMI1640 containing 11 mM glucose for 24 h, and [Ca^{2+}]i was measured with and without 50 mM KCl treatments as described under Materials and Methods. Data are mean ± S.E. (n).

ability of the N-type Ca^{2+} channel, but not that of the L-type Ca^{2+} channel. Although basal $[Ca^{2+}]_i$ reported here is slightly lower [~30 nM] than values reported in pancreatic β cells (38,39), it may reflect the characteristics of βTC 3 cells. Thus, the data indicate that syntaxin 1A expressed in pancreatic β cells is not associated with Ca^{2+}-channel, suggesting that the exocytosis machinery of insulin release is different from that of neurotransmitter release.

In conclusion, syntaxin 1A plays a crucial role in the regulated pathway of insulin release, particularly in late step of exocytosis, without mediation by glucose metabolism and $[Ca^{2+}]_i$ in insulinoma cell line βTC3 cells.

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