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SNAP-25 Is Phosphorylated by Glucose and GLP-1 in RIN 1046-38 Cells

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We investigated the possibility that tyrosine phosphorylation might play a role in insulin secretion in the insulinoma cell line, RIN 1046-38 cells. At least 4 proteins of 18, 25, 35, and 46 kDa size were found to be tyrosine phosphorylated in the presence of glucose and an insulin secretagogue, glucagon-like peptide-1 (GLP-1). The addition of glucose and GLP-1 to cells that were exposed to the tyrosine kinase inhibitor genistein resulted in a decrease in the extent of phosphorylation of the 18, 25, and 35 kDa proteins and a concomitant reduction in insulin secretion, whereas treatment with vanadate, a tyrosine phosphatase inhibitor, led to enhanced responses. Immunoprecipitation of cellular proteins with an antiphosphotyrosine antibody followed by immunoblotting with a specific monoclonal antibody to SNAP-25 (synaptosome-associated protein of 25 kDa) revealed that the 25 kDa protein is SNAP-25. These results suggest that tyrosine phosphorylation of SNAP-25 may be involved in the regulation of insulin secretion in RIN 1046-38 cells.

Insulin is stored in beta cells in large dense-core secretory vesicles (LDCVs) that accumulate in proximity of release sites and fuse with the plasma membrane in response to secretagogues (1). The molecules involved in neuronal synaptic vesicle exocytosis have been characterized (2); however, little is known about exocytosis in endocrine cells and it is just lately that some information has become forthcoming (3, 4). It appears that beta cells and neurons share similar molecular mechanisms for exocytosis (4). At the point of synapsis, vesicle-associated membrane protein (VAMP) of the vesicle membrane interacts with two presynaptic plasma membrane proteins; the synaptic-associated protein of 25 kDa (SNAP-25) and syntaxin (5). Together these three molecules form a complex which functions as a SNAP receptor, also known as SNARE. SNAPs (soluble NSF attachment proteins) and NSF (*N*-ethylmaleimide-sensitive factor) bind to the SNARE at the vesicle/target membrane interface, resulting in the fusion of both the vesicular and plasma membranes followed by exocytosis (6). Under certain circumstances, exocytosis from vesicles is calcium-dependent (7). Posttranslational modification of proteins may also alter protein function. Indeed, the state of phosphorylation of SNAP-25 appears to regulate catecholamine release (8), and possibly other hormones.

In acinar cells of the pancreas, it has been shown that tyrosine phosphorylation events are involved in the regulation of amylase release by cholecystokinin (9, 10). The nature of these signalling proteins are unknown but may involve phosphorylation of either VAMP or the plasma membrane proteins involved in fusion and/or exocytosis of amylase. Both endocrine and exocrine cells of the pancreas are derived from a common progenitor cell (11, 12) and both contain LDCVs for the regulated release of insulin and amylase, respectively. These observations led to the postulate that tyrosine phosphorylation events might be involved in insulin release. The aim of this study was to explore the role of tyrosine phosphorylation in the regulation of insulin release by glucose and the insulin secretagogue, GLP-1, in an insulinoma cell line, RIN 1046-38.

MATERIALS AND METHODS

Materials. All chemicals used were of the highest purity available. Medium 199 (M199) was from the NIH Media Unit. Fetal bovine serum (FBS) and trypsin/EDTA solution were purchased from Gibco BRL (Grand Island, NY). GLP-1, glucagon and insulin were purchased from Bachem (Torrence, CA). Monoclonal and polyclonal antiphosphotyrosine antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY) and protein G Plus/protein A agarose beads from Oncogene Science, Inc. (Uniondale, NY). Monoclonal antibody (Clone #SP12) against SNAP-25 was from Serotec Co. (Oxford, England). Enhanced chemiluminescent (ECL) detection system, horseradish peroxidase conjugated donkey anti-rabbit IgG, sheep anti-mouse IgG and ^{125}-I-insulin kit were from Amersham Co. (Arlington Heights, IL). Polyvinylidene difluoride (PVDF) membrane and precast gradient 4-20% polyacrylamide gels were from Novex (San Diego, CA). Tween-20, Triton X-100, phenylmethylsulfonyl fluoride (PMSF), genistein and Na-orthovanadate were from Sigma

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Chemical Co. (St. Louis, MO). Cells were from American Type Culture Collection (Rockville, MD). Benzamidine, leupeptin and aprotinin were obtained from ICN (Costa Mesa, CA). Protein assay reagent was from BIO-RAD Laboratories (Hercules, CA).

Cell culture and treatment. RIN cells were propagated in culture conditions, as previously described (13). In brief, cells were grown to approximately 80% confluency in M199, 5% heat-inactivated FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 2 mM glucose at 37°C with 95% air-5% CO_2. They were then washed twice with Kreb-Ringer's balanced salt solution (KRB) without glucose and were preincubated in KRB for 2 h at 37°C. Then the culture dishes were transferred to an aluminium cooling plate (Pharmacia) connected to a thermostatic water circulator (Lauda). After a 5-min incubation at 37°C, the phosphorylation reaction was carried out by adding 20 mM glucose and/or 10 nM GLP-1. In some cases, the cells were treated with the tyrosine kinase inhibitor genistein or the tyrosine phosphatase inhibitor vanadate for 30 min prior to addition of glucose and GLP-1. The incubation medium was collected after 2 min, and the cells rapidly washed before their immersion in liquid nitrogen. The collected media were centrifuged (2 min, 2,000 *xg*, 4°C) and the supernatants stored at −20°C until further analysis for insulin content by RIA.

Immunoprecipitation, SDS-PAGE, and immunoblotting. The frozen cells were scraped and lysed in the immune precipitation buffer containing 25 mM Tris-HCl (pH 8.0), 137 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.2 mM PMSF, 1 mM Na-orthovanadate, 1 mM benzamidine, 10 μg/ml leupeptin and 20 μg/ml aprotinin. Insoluble material was removed by centrifugation at 15,000 *xg* for 15 min, and the clarified lysates were incubated with a monoclonal anti-phosphotyrosine antibody or monoclonal anti-SNAP-25 antibody at 4°C overnight with constant agitation. Protein G Plus/protein A agarose beads were added and 4 h later the immunocomplexes were washed three times with the immune precipitation buffer and once with washing buffer (25 mM Hepes [pH7.4], 0.1% Triton X-100 and 1 mM Na-orthovanadate). The samples were denatured in Laemlli sample buffer (14) in the presence of 10% 2-mercaptoethanol by heating at 100°C for 5 min, and then resolved by electrophoresis on 4-20% or 12% precasted gradient polyacrylamide gel. After electrophoresis, proteins were electrotransferred to PVDF membrane. The PVDF membranes were blocked with 5% (w/v) non-fat dry milk in TBST buffer (20 mM Tris-HCl [pH 7.5], 137 mM NaCl and 0.1% Tween 20) for 1 h at room temperature, and then incubated with antibody to SNAP-25 (1: 5,000 dilution) or polyclonal anti-phosphotyrosine (1:1,500) for 1 h at room temperature. PVDF membranes were washed three times with TBST and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antisera for 1 h at room temperature. After a series of washes in TBST, the blots were developed using the ECl chemiluminescent detection system. Autoradiographs were quantified by using Image-Quant software (version 3.3) on a Molecular Dynamics laser densitometer. Aliquots (20 μl) of clarified cell lysates were used to determine protein concentration which was estimated by the Bradford method with bovine gamma globulin as standard.

Statistical analysis. All data are expressed as mean ± SEM. A value of $p < 0.05$ by non-paired Student's t-test was considered significant.

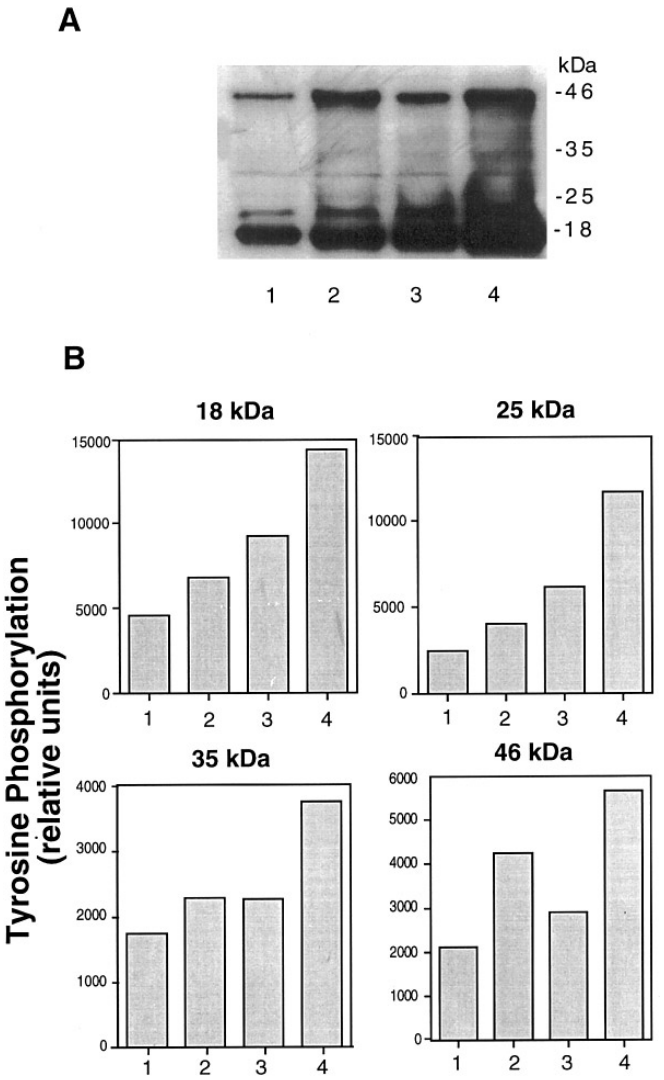

FIG. 1. Protein tyrosine phosphorylation in RIN 1046-38 cells in response to glucose and GLP-1. (A) RIN cell lysates were immunoprecipitated with a monoclonal antibody to phosphotyrosine, and the immunoprecipitated complexes were subjected to SDS-PAGE and transferred to PVDF membrane. The blot was probed with polyclonal anti-phosphotyrosine antibody and detected using a chemiluminescent technique. Cells were treated for 2 min in the following manner: (1) no treatment, (2) glucose alone (20 mM) (3) GLP-1 alone (20 nM), and (4) glucose + GLP-1. (B) densitometric quantification of selected bands from the gel in A.

RESULTS AND DISCUSSION

To determine if glucose is able to enhance tyrosine phosphorylation of cellular proteins in RIN cells, we incubated lysates from glucose-treated cells with a monoclonal anti-phosphotyrosine antibody followed by Western blot analysis with polyclonal anti-phosphotyrosine antibody (Fig. 1). An increase in phosphorylation of proteins at 18 (1.5-fold), 25 (1.65-fold), 35 (1.3-fold), and 46 (2.0-fold) kDa was observed when compared to untreated cells. Likewise, GLP-1 caused an increase in the phosphorylation of the same proteins but with a different pattern. While GLP-1 treatment lead to a 2.5-fold increase in phosphorylation of the 18 and 25 kDa proteins phosphorylation of the 35 and 46 kDa proteins was increased 1.3-fold only. Both glucose and GLP-1 had additive effects on the 4 proteins of interest.

Next, we analysed the antiphosphotyrosine immuno-

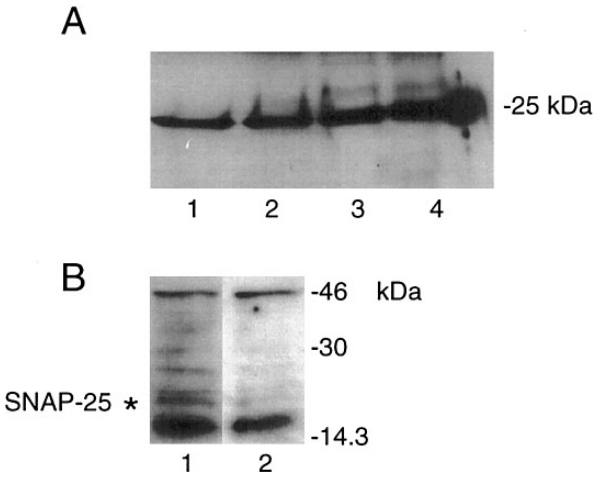

FIG. 2. Effects of insulin-secreting agents on tyrosine phosphorylation of SNAP-25. (A) RIN 1046-38 cells were treated in the following manner: (1) no treatment, (2) glucose alone (20 mM), (3) GLP-1 alone (20 nM), and (4) glucose + GLP-1 for 2 min. Cells were lysed and the phosphoproteins were immunoprecipitated with a monoclonal anti-phosphotyrosine antibody. The immunoprecipitates were separated by SDS-PAGE and the proteins were electrotransfered to PVDF membrane. The membrane was immunoblotted with a monoclonal anti-SNAP-25 antibody. (B) Alternatively the PVDF membrane was incubated with an anti-phosphotyrosine antibody in the absence (1) or presence of (2) 1000-fold excess phosphorylated tyrosine.

precipitate with an anti-SNAP-25 antibody (Fig. 2A). The fact that a 25 kDa phosphoprotein co-migrated with SNAP-25 may indicate that SNAP-25 is phosphorylated. To verify the specificity of the tyrosine phosphorylation PVDF membrane was reprobed with anti-phosphotyrosine antibody in the presence of 1,000-fold excess phosphorylated tyrosine. The intensity of the signal associated with the 18 kDa protein markedly decreased while that of the 46 kDa band was not affected. Furthermore, the bands at 25 and 35 kDa as well as other lesser bands were abolished (Fig. 2B).

To further confirm that tyrosine phosphorylation events occurred following cell treatment with glucose and/or GLP-1, the effect of a chemical inhibitor of tyrosine kinase was assessed. Genistein at 300 and 500μM (but not 100 μM) inhibited phosphorylation of all the phosphorylated proteins in a concentration-dependent manner (Fig. 3A). These concentrations of genistein also decreased glucose- and GLP-1-mediated insulin secretion (Fig. 3B). However, even though genistein at 300 μM totally abolished insulin secretion in response to secretagogues, the proteins of interest were still phosphorylated to a greater degree than non-stimulated cells. The implication is that other proteins involved in insulin secretion are also not being activated in the presence of genistein. Vanadate (200 μM), a tyrosine phosphatase inhibitor, potentiated glucose- and GLP-1-mediated tyrosine phosphorylation and potentiated insulin secretion in response to secretagogues (Fig. 4 A,B). The 18 and 35 kDa proteins were immunoprecipitated also by the anti-SNAP-25 antibody. These match the size of VAMP (18 kDa) and syntaxin (35 kDa) (15,16) and probably indicates that phosphorylation activates SNAP-25 and allows its association with the other two SNARE proteins, syntaxin and VAMP. This complex formation as has already been described in neurons and beta cells (17, 18).

In this report, we have demonstrated that; 1) SNAP-25 is phosphorylated on tyrosine; 2) tyrosine phosphorylation events occur in response to glucose and GLP-1; and, 3) an increase in tyrosine phosphorylation by vanadate and a decrease by genistein altered insulin

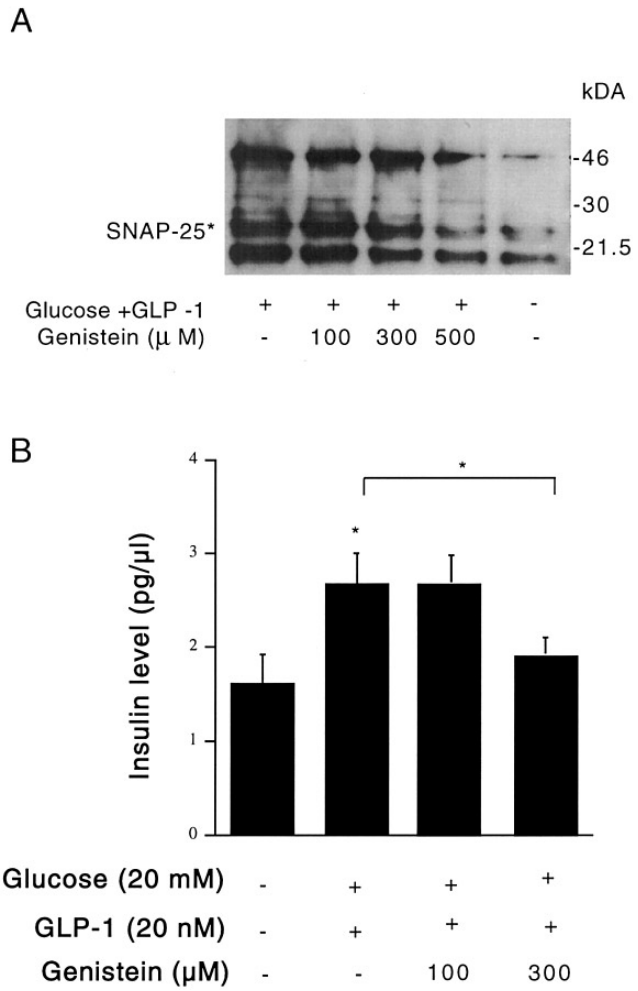

FIG. 3. Effects of genistein on SNAP-25 tyrosine phosphorylation and insulin secretion in RIN 1046-38 cells. (A) Cells were pre-incubated with genistein for 30 min prior to the addition of the secretagogues. Two min later, the media were collected for the measurement of insulin content (B). (A) Cells were lysed and a monoclonal anti-SNAP-25 antibody was added to the clarified cell lysates. The immunoprecipitates were separated by SDS-PAGE and the proteins electrotransferred to PVDF membrane which was immunoblotted with antiphosphotyrosine antibody. Values represent means ± SEM of three experiments. *Indicates significant difference ($p<0.05$).

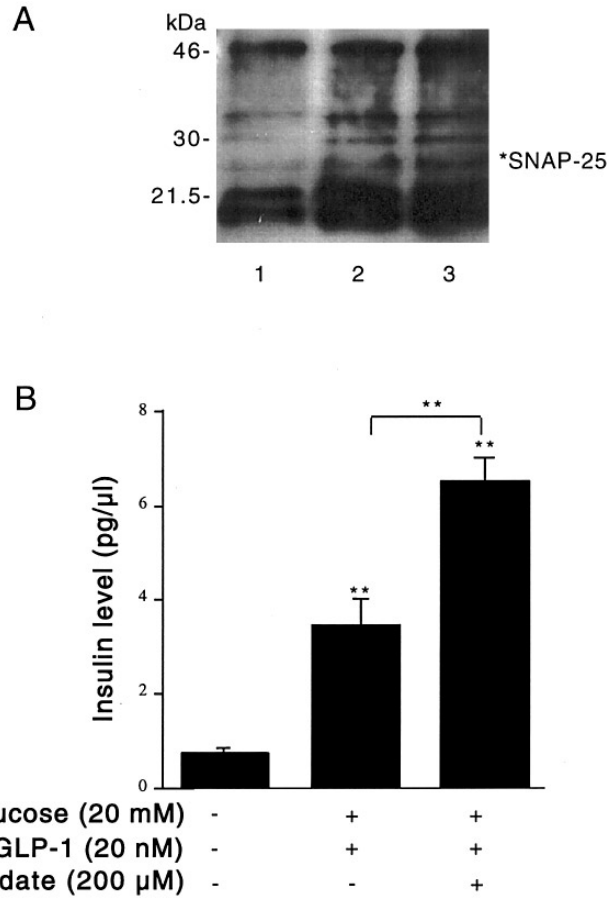

FIG. 4. Effects of vanadate on SNAP-25 tyrosine phosphorylation and insulin secretion in RIN 1046-38 cells. Cells were pre-incubated with vanadate for 30 min followed by a 2 min treatment with the secretagogues. At the end of the treatment, media were collected for the measurement of insulin content (B). (A) Cells were lysed and a monoclonal anti-SNAP-25 antibody was added to the clarified cell lysates. The immunoprecipitates were separated by SDS-PAGE and the proteins electrotransferred to PVDF membrane and immunoblotted with anti-phosphotyrosine antibody. Values represent means ± SEM of three experiments. **Indicates significant difference ($p<0.01$).

secretion. To our knowledge this is the first time that tyrosine phosphorylation on SNAP-25 has been shown to occur. Serine phosphorylation has been shown on Ser^{187} in response to protein kinase C in PC12 cells (8). Pancreatic SNAP-25 (called SNAP-23) is a different isoform from neuronal SNAP (19). It contains an additional tyrosine at position 139 (both SNAP-25 and -23 have a tyrosine at position 99), so this is possibly being phosphorylated in RIN cells.

Insulin secretion is controlled by various signal transduction systems. Protein kinase and phosphatases would appear to play an important role in these events. Although the mechanism that promotes phosphorylation of SNAP-25 and other proteins by GLP-1 and glucose remains to be resolved, tyrosine phosphorylation of SNAP-25 appears to be involved in regulated insulin secretion in RIN 1046-38 cells.

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