Rapid communications

Role of syntaxin in mouse pancreatic beta cells

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Summary The role of syntaxin 1, a protein involved in the docking of synaptic vesicles at presynaptic active zones, has been investigated in pancreatic islet cells. Using two different monoclonal antibodies we have shown that syntaxin 1 is present in the pancreatic islet cell microsomal fraction. Furthermore, functional experiments demonstrate that anti-syntaxin antibodies inhibit Ca^{2+} -dependent insulin secretion

in permeabilized islet cells. These data indicate that syntaxin 1 is present in the pancreatic beta cell and it is likely to play a functional role in the exocytosis of secretory granules. [Diabetologia (1995) 38: 860–863]

Key words Exocytosis, syntaxin, pancreatic beta cell, secretion, SNAP receptor.

Changes in intracellular free calcium concentration ($[\text{Ca}^{2+}]_i$) in pancreatic beta cells play an important role in the regulation of insulin secretion. In nerve terminals a basal increase of $[\text{Ca}^{2+}]_i$ triggers the fusion of a portion of the synaptic vesicle population with the presynaptic plasma membrane, resulting in neurotransmitter release. The processes of vesicle docking, activation and fusion are likely to involve protein components localized both in the secretory vesicle and in the plasma membrane (SNARE hypothesis) as well as soluble factors, which have been recently described. These proteins include synaptotagmin and synaptobrevin/VAMP (vesicle-associated membrane protein) in the vesicle membrane and syntaxin and soluble synaptosomal associated

protein (25 kD) (SNAP-25) in the presynaptic membrane [1]. As the SNARE hypothesis mentioned above offers an universal model for the docking of vesicles to their target membranes, it is important to test this model for non-neuronal cell types. In this regard, recent studies show that like other peptide-secreting endocrine cells, pancreatic endocrine cells are positive for several synaptic proteins, including VAMP, SNAP-25, syntaxin, munc-18 [2], synaptobrevin, synaptophysin, protein p29, SV2 and rab3A [3]. In this study we have examined the presence and the functional role of syntaxin 1 in pancreatic islet cells using specific monoclonal antibodies.

Materials and methods

Materials. Monoclonal anti-syntaxin (clone HPC-1), anti-mouse IgG peroxidase conjugate and mouse IgG were from Sigma (St. Louis, Mo., USA). The monoclonal anti-syntaxin antibody used in the immunoblotting test (mAb 44D5) was isolated in a screening of neurospecific antigens expressed in *Drosophila* central nervous system [4]. Electrophoresis reagents were from BioRad (Richmond, Calif., USA). Collagenase was from Boehringer Mannheim (Mannheim, Germany). RIA kit was from Novo Nordisk (Bagsvaerd, Denmark). Immobilon polyvinylidene difluoride (PVDF) membranes were from Millipore-Iberica (Barcelona, Spain). Tis-

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Abbreviations: $[\text{Ca}^{2+}]_i$, intracellular free calcium concentration; PMSF, phenyl methyl sulphonyl fluoride; PVDF, polyvinylidene difluoride; SNAP, synaptosomal associated protein; SNARE, SNAP receptor; VAMP, vesicle-associated membrane protein; mAb, monoclonal antibody; clone HPC-1, anti-syntaxin monoclonal antibody.

sue culture reagents were obtained from Cultek (Madrid, Spain).

Cell isolation and subcellular preparations. Adult (8–10-week-old) male Swiss mice (OF1) (CRIFFA, Barcelona, Spain) were used throughout this study. Pancreatic islet cells were dispersed from isolated islets as previously described [5]. Pancreatic islet cell membranes were prepared from 100 isolated islets of Langerhans. Isolated islets were sonicated twice in 50 mmol/l Tris/CIH pH 7.2 buffer, containing 1 mmol/l phenyl methyl sulphonyl fluoride (PMSF), 1 mmol/l EGTA, for 5 s using a Sonic dismembrator (Artek System Corp., Farmingdale, N.Y., USA) at 30 % maximal power. The suspension was finally centrifuged for 30 min at $150,000 \times g$ in a Beckman TL100 (Beckman Corp., Palo Alto, Calif., USA). Proteins in the sediment were analysed by 1D-PAGE. Synaptosomes from rat brain cortex were prepared in Ficoll gradients according to the method of Cotman [6].

Electrophoresis, electroblotting and immunodetection. Protein samples were separated on 10 % SDS-PAGE, transferred onto PVDF membranes and analysed by Western blotting using anti-syntaxin clone HPC-1 at a dilution of 1:1000 and anti-syntaxin mAb44D5 at a dilution of 1:100. The antibodies were detected by incubating blots with anti-mouse IgG peroxidase conjugated at a dilution of 1:1000.

Immunocytochemistry. Pancreatic islet cells (2.5×10^5) were incubated with the monoclonal anti-syntaxin antibody clone HPC-1 at a dilution of 1:200 and mAb44D5 at a dilution of 1:50 for 24 h; after washing, cells were incubated with the peroxidase-labelled secondary antibody at a dilution of 1:200 for a further 2-h period. Finally, cells were washed in 50 mmol/l Tris/CIH pH 7.2 buffer, containing 500 mmol/l NaCl, and colour was developed using 1,2 diaminobenzidine. Control cells were treated in the same way using normal mouse immunoglobulins as first antibody. Cells were visualized and photographed using a Zeiss Chasis Axiomat 35-mm camera coupled to a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany).

Cell permeabilization and insulin secretion. Pancreatic islet cells (4×10^5) were permeabilized by incubation with 10 μ mol/l digitonin, at 37°C, for 10 min, in Hepes buffer, pH 7.0 (in mmol/l) (25 Hepes, 110 KCl, 10 NaCl, 2 KH_2PO_4 , 1 MgCl_2 , 5 succinate and 1 mg/ml bovine serum albumin). During the permeabilization period islet cells were incubated in the absence or presence of the different antibodies. After permeabilization islet cells were centrifuged and resuspended in the same Hepes buffer supplemented with 2 mmol/l MgATP and an ATP-regenerating system consisting of 15 mmol/l phosphocreatine and 20 U/ml creatine kinase. Insulin release from permeabilized islet cells was studied by incubating cells in the supplemented Hepes buffer with two different concentrations of free Ca^{2+} , 50 nmol/l (basal secretion) and 10 μ mol/l (stimulated secretion), with or without presence of the different antibodies assayed. In this case 5 mmol/l EGTA and Ca^{2+} in the appropriate amounts to give the desired Ca^{2+} concentrations was added to the supplemented Hepes buffer. The permeabilized islet cells were incubated with gentle agitation for 10 min at 37°C. After incubation cell suspensions were centrifuged and samples were taken from the supernatant for insulin determination by RIA.

Statistical analysis

Data are expressed as mean $\pm$ SEM. Student's *t*-test was used to determine statistical significance ($p < 0.05$).

Results and discussion

Detection of syntaxin 1 in pancreatic islet cells. Figure 1A shows that monoclonal antibody mAb44D5 recognized a single major band of 35 kDa in the fraction containing pancreatic islet cell membrane proteins (lane 1) and in the fraction containing total pancreatic islet cell proteins (lane 3). No positive labelling for syntaxin 1 was observed with mAb44D5 in the pancreatic islet cell cytosolic fraction proteins (lane 2) or in a $100,000 \times g$ rat liver membrane microsomal preparation used as control (lane 5). Finally, synaptosomes from rat brain cortex also showed the 35 kDa band labelled with mAb44D5 (lane 4). Similar results were obtained using the anti-syntaxin monoclonal antibody (clone HPC-1) (data not shown). In order to confirm that syntaxin 1 is present in pancreatic islet cells, we used an immunocytochemical approach. Figure 1B shows that cultured pancreatic islet cells were labelled with antibody HPC-1, but not in control conditions, in which normal mouse immunoglobulins were used. Similar results were observed with mAb44D5. Thus, as has been recently demonstrated [2], we confirm by two methodological approaches (Fig. 1), the presence of syntaxin 1 in pancreatic islet cells. Moreover, immunoblotting for syntaxin 1 across subcellular fractions of pancreatic islet cells (Fig. 1A), also indicates that syntaxin 1 is present in the microsomal membrane fraction. Thus, it appears that localization of syntaxin 1 in pancreatic islet cells is similar to its localization in the presynaptic terminals [2]. Different members of the syntaxin family have been described in the rat [7], each of them with a particular tissue distribution; the antibodies used in this study have been shown to recognize only either one or both isoforms (1a and 1b) of the neuronal specific member of the syntaxin family, which is syntaxin 1 [4, 8].

Inhibitory effect of anti-syntaxin on pancreatic islet cell secretion. Figure 2 shows that clone HPC-1 at a dilution of 1:250 in which maximal effect was observed and mAb44D5 at a dilution of 1:50 which is in the range of previous studies [4] provoked an inhibition of Ca^{2+} -dependent insulin secretion in digitonin-permeabilized pancreatic islet cells. The inhibitory effect was 54 % ($p < 0.05$) with the clone HPC-1 and 26 % ($p < 0.05$) with mAb44D5. Basal insulin release was not affected ($p = \text{NS}$) by the presence of the anti-syntaxin monoclonal antibodies (clone HPC-1 and mAb44D5) (Fig. 2). To demonstrate the specific of the inhibition of the anti-syntaxin antibodies, mon-

Fig. 2. Effect of the anti-syntaxin monoclonal antibodies on insulin secretion in digitonin-permeabilized pancreatic islet cells. Two anti-syntaxin monoclonal antibodies (clone HPC-1 and mAb 44D5) were incubated for 10 min in the presence of the digitonin-permeabilization solution as indicated in Methods, then medium was removed and secretion was induced for 10 min in the presence of 50 nmol/l Ca²⁺ (basal secretion) or 10 µmol/l Ca²⁺ (stimulated secretion), both in the absence or the presence of each monoclonal antibody. Monoclonal antibodies heated at 100°C for 2 h were also tested in the same conditions as a control at the same dilutions. A and D, cells incubated in the absence of antibodies; B and E, cells incubated in the presence of anti-syntaxin clone HPC-1 (dilution 1:250); C and F, cells incubated in the presence of anti-syntaxin mAb 44D5 (dilution 1:50); G, cells incubated in the presence of anti-syntaxin done HPC-S (dilution 1:290) heated at 100°C for 2 h; H, cells incubated in the presence of anti-syntaxin mAb 44D5 (dilution 1:50) heated at 100°C for 2 h. Results are mean ± SEM from five separate experiments. * $p < 0.05$, when compared with D; G and H vs D, $p = \text{NS}$. B and C vs A, $p = \text{NS}$

oclonal antibodies heated at 100°C for 2 h were used as control. The same dilutions of both monoclonal antibodies had no effect on Ca²⁺-dependent insulin release. Thus, we show for the first time that syntaxin 1 is implicated in the calcium-dependent secretory pathway, and thus involved in the control of exocytosis of the insulin-containing secretory granules (Fig. 2). These results indicate that syntaxin 1 is an important element of the Ca²⁺-dependent secretory machinery in pancreatic beta cells, most probably associated with a high molecular weight "fusion complex" as has been previously proposed for synaptic vesicles [1]. Similar results have recently been shown in permeabilized chromaffin cells [9]. There are several possible reasons that could explain why the inhibition was not complete. Dose-dependent studies of the inhibition of Ca²⁺-dependent insulin secretion by the antibody HPC-1, showed that the inhibition was maximum at an immunoglobulin dilution of 1:250 (data not shown). The fact that the inhibition did not further increase significantly with higher concentrations of the antibody, suggests that the accessibility of the antibody is probably not the reason for this partial inhibitory effect. The antibody could be affecting the docking of a population of granules that has not yet reached the membrane, resulting in a partial inhibition; granules already docked at the membrane and possibly forming an active complex would not be affected. In this regard, it has been recently suggested that the exocytotic machinery of the beta

cell is capable of operating at very high rates (4–5 % of the total granule population per second) [10].

Insulin secretory granules are large dense core vesicles different from synaptic vesicles, thus, this study supports the concept that common mechanisms may underlie a wide variety of vesicle-mediated secretory processes. It seems possible that the differences concerning the time needed for maximal secretion between the synaptic cleft and endocrine cells may simply be due to the fact that the hormone-containing vesicles are 5–10 times larger than the synaptic vesicles and to the high concentration of vesicles accumulated and docked at the active zones of the synaptic terminals. Additionally, differences in the soluble factors participating in the fusion process in endocrine and neuronal systems can be important in the regulation of the secretory process.

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