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Engineered Peptides Corresponding to Segments of the H3 Domain of Syntaxin Inhibit Insulin Release both in Intact and Permeabilized Mouse Pancreatic β Cells

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Syntaxin is one of the proteins involved in the exocytotic event through sequential binding to specific proteins, including SNAP25 and synaptobrevin. In a previous work in digitonin-permeabilized β cells, we characterized the functional role of two segments: synA and synB of the H3 domain of syntaxin. As a continuation of these experiments in the present study we have initially outlined a zone of 17 residues as the very effective uncoupling element of the synA segment. Further functional studies have been accomplished in intact pancreatic β cells with a specific myristoylated (myr) 13-mer peptide comprised in this active zone. These experiments showed a concentration-dependent inhibition of glucose-induced insulin release (IC_{50}=4 μM) of this engineered peptide that was specific since a myristoylated random peptide with the same composition was ineffective. A second myristoylated 13-mer peptide comprised into the synB segment was shown to be even more potent promoting a selective inhibition of insulin release. These data show for the first time, that nutrient-induced secretory process can be specifically uncoupled in intact β cells demonstrating at the same time that syntaxin plays a central role in this mechanism.

Key Words: **syntaxin; exocytosis; myristoylation; insulin release.**

Proteins implicated in the last stages of the secretory pathway which allow targeting and fusion of vesicles, were initially characterized in the synapsis terminal (1,2). However, further studies have also demonstrated the presence and the active role of these proteins in endocrine systems (3-5).

Syntaxin, in the plasma membrane, is one of the proteins of the secretory complex that plays a pivotal role, interacting in a probable sequential mechanism with other proteins during docking, priming, activation and fusion of secretory granules. SNAP25, also in the plasma membrane, has been demonstrated to interact with syntaxin through specific domains allowing a more effective association with the two specific counterpart proteins in the synaptic vesicle, VAMP, also called synaptobrevin, and synaptotagmin (6). Soluble proteins as α-SNAP and NSF also participate in the process, although their precise role is still being studied (7).

Because of the central role that syntaxin displays in regulated secretion, a special effort has been recently dedicated to understand which specific domains of this polypeptide could be directly implicated in protein-protein interactions during the secretory process. In the molecule of syntaxin, three domains (H1, H2 and H3) have recently been characterized according to their hyphotetic α-helical conformation (8) which could participate in specific interactions through their coiled-coil motifs. The H3 region is probably the most interesting because is the most highly conserved and besides *in vitro* studies show its participation in the binding of other components of the fusion complex (8). Sequence analysis of the H3 domain shows the presence of characteristic helical heptads in which *a* and *d* positions are regularly occupied by hydrofobic residues.

In a previous study (9) we described two peptides, syn-A and syn-B, corresponding to two segments of H3 domain, which inhibited specifically calcium-induced insulin release in permeabilized mouse pancreatic β cells.

A recent approach using myristoylated peptides has allowed to overcome the permeability barrier of the plasma membrane and therefore, it supposes an inter-

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Abbreviations used: SNAP-25, synaptosomal-associated protein of 25 kDa; VAMP, vesicle-associated membrane protein; NSF, N-ethylmaleimide-sensitive fusion factor; SNAP, soluble NSF attachment protein.

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esting possibility to study the participation of specific protein domains in different processes by using structurally related peptides in intact cells. This methodology has already been successfully used to study the role of protein kinase C (10) and protein kinase A (11,12) in different regulatory mechanisms. In the present study we have demonstrated that 13-mer myristoylated peptides corresponding to the H3 domain of syntaxin are able to uncouple the nutrient- induced secretory process in intact pancreatic β cells in the micromolar range.

MATERIALS AND METHODS

Materials. Collagenase was from Boehringer-Mannheim (Mannheim, Germany). Tissue culture reagents were from Cultek (Madrid, Spain).

Cell isolation, permeabilization and insulin secretion. Adult (8-10 week-old) male swiss mice (OF1) (CRIFFA, Barcelona, Spain) were used throughout this study. Pancreatic β cells were dispersed from isolated islets and cultured as previously described (9). Experiments with permeabilized cells were carried out by using 10 μM digitonin as previously described (5). Experiments with intact cells were carried out in presence of a Krebs-bicarbonate salt solution, pH7.4, suplemented with 1% BSA and 3 mM glucose in which the different peptides were incubated at 37 °C for 2h before the stimulus that was carried out for 30 more min with 22 mM glucose.

Peptide synthesis, purification, and myristoylation. Peptides were synthesized using standard Fmoc chemistry in an ABI431A peptide synthesizer. After the last coupling step, the resin was divided in two portions. One was used to produce the unmodified peptide, and the second portion was subjected to myristoylation (10). After deprotection of the amino terminus of the immobilized peptide, myristic acid (10 equivalents) was activated with DCC (N,N-dicyclohexylcarbodiimide) and HOBt (1-hydroxybenzotriazole) in NMP (N-methylpyrrolidone), transferred to the resin and incubated for 30 min. The peptide was then washed and deprotected according to the standard protocol. Purity of the peptides was assayed by RP-HPLC, and peptide identity was checked by electrospray-ion trap mass spectrometry using a Finnignan LCQ (Thermoquest, USA). The sequence of the synthesized peptides is represented in Figure 1A: A series of six 13-mer peptides corresponding to synA were sythesized in order to be used in permeabilized cells, S1 (MIDRIEYNVEHAV), S2 (DRIEYNVEHAVDY), S3 (IEYNVEHAVDYVE), S4 (YNVEHAVDYVERA), S5 (VEHAVDYVERAVS) and S6 (HAVDYVERAVSDT). The sequences of myristoylated peptides used in experiments carried out in intact β cells are the following: myr-syn$A_{233\text{-}245}$ (IEYNVEHAVDYVE) and its corresponding control with aleatory sequence, myr-conA (YVVEEDNHIYEAV) and myr-syn$B_{200\text{-}212}$ (SEIIKLENSIREL) and its control with aleatory sequence, myr-conB (NLKIREEISLSIE). Non-myristoylated peptides with the same sequences but lacking the N-terminal myristate were also synthesized and used in control experiments: syn$A_{233\text{-}245}$, syn$B_{200\text{-}212}$, conA and conB.

Statistics. Results are presented as means ± S.E.M. Statistical analysis was performed by Student's two tailed *t* test for unpaired data. $*p < 0.001$ and $**p < 0.0002$ were considered to be significant.

RESULTS AND DISCUSSION

Studies in permeabilized cells. Recently, we have reported that two 23-mer peptides, synA and synB, corresponding to two regions of the H3 domain of syntaxin, residues 229-251 and 197-219 respectively, inhibited

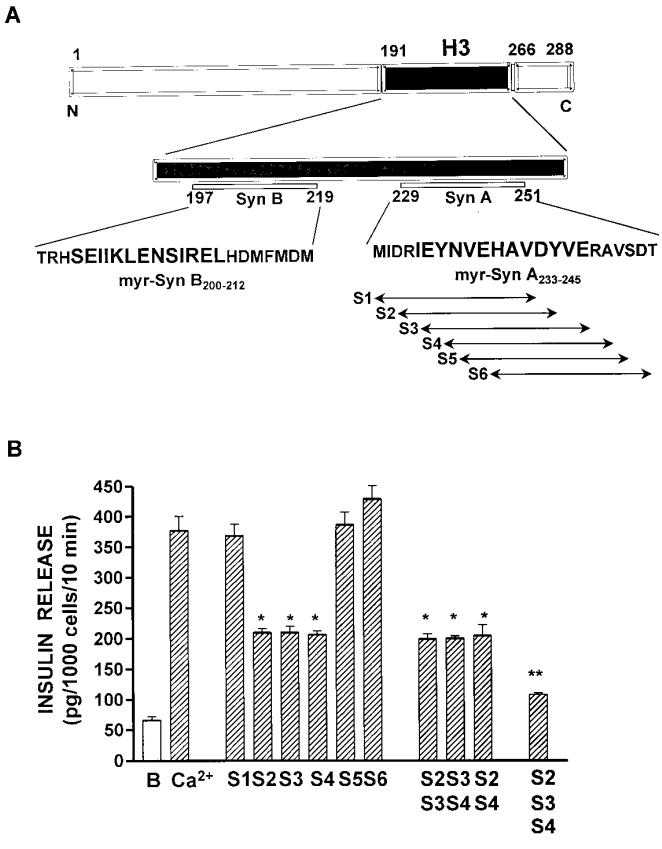

FIG. 1. Functional implications of H3 domain on insulin release in permeabilized β cells. A. Schematic diagram of the syntaxin 1A protein including the H3 domain and the segments synA and synB. The sequences of the myristoylated peptides used in intact cells are outstanding in bold characters. B. Effect of a series of 13-mer peptides (S1-S6) spanning the synA segment on calcium-induced insulin release. Digitonin-permeabilized β cells were incubated at 37 °C with 200 μM of the different peptides for 5 min before the stimulus in the presence of 10 μM Ca^{+2} for 10 additional min. IRI, immunoreactive insulin. Experimental data are expressed as means ± SEM of 3 experiments performed in triplicate. $*p < 0.001$ and $**p < 0.0002$ when compared with 10 μM Ca^{2+} stimulus.

calcium-induced insulin release in permeabilized β cells (9). In order to characterize the minimal segment that could be involved in this effect, we have synthesized a series of six 13-mer peptides (S1-S6) that sequentially and with partial overlapping span the entire region of synA (Figure 1 A). In order to study the effect of these peptides on calcium-induced insulin release, functional experiments were carried out in digitonin-permeabilized β cells which were incubated with 200 μM final concentration of the different peptides for 5 min before the addition of the secretory stimulus in presence of 10 μM Ca^{+2} which was applied for an additional 10 min period. Figure 1 B shows the inhibitory effect of peptides S2, S3 and S4. In all cases the inhibition over the calcium-induced release was significant ($*p < 0.001$) reaching 45%. The other peptides of the series S1,S5 and S6 had any significative effect. With

binary combinations the inhibitory effect was roughly the same (*$p < 0.001$), however the utilization of the three peptides S2, S3 and S4 together caused a further effect resulting almost 75% inhibition (**$p < 0.0002$), that is the same range of blockade we observed with the entire synA. These experiments allow us to outline a central region of 17 residues into synA, comprised by these three functional active peptides, as the minimum domain, in this region of the protein, able to uncouple calcium-induced secretion in permeabilized β cells.

Studies in intact cells. In order to study the possibility of uncoupling specifically the secretory process in intact cells, we have synthesized two N-terminal-myristoylated peptides included into the two segments of H3, synA and synB previously referred, having a potential relevance in secretion. In previous studies, myristoylated peptides with nine residues (10) or even longer sequences have been successfully used in intact cells (12). We have intended to synthesize two myristoylated peptides having in their sequence at least four residues in *a* and *d* sites of the hypothetical α-helix, taken into consideration a balance between possible intracellular activity and membrane permeability. Accordingly, we have initially synthesized a myristoylated 13-mer peptide, myr-syn$A_{233\text{-}245}$ comprised into the active zone of synA previously characterized. Concerning the sequence of synB, it has recently been reported that a 32-mer peptide, corresponding to residues 189-220, which includes the 23-mer synB segment, is able to inhibit SNAP-25 binding to syntaxin, besides this binding is reduced after puntual mutations in residues 205 and 209 that would occupy *a* and *d* sites of the helix (13). These data prompted us to prepare a second myristoylated 13-mer peptide, myr-syn$B_{200\text{-}212}$, including these apparently important residues into its central core, having a high possibility to uncouple secretion. Both myristoylated peptides were incubated at 37 °C for 2 h in 3 mM basal glucose before to the stimulus which was accomplished by incubating β cells with 22 mM glucose for 30 more min. Figure 2 shows the dose-dependent inhibitory effect of both myristoylated peptides on glucose-induced insulin release, the IC_{50} of myr-syn$B_{200\text{-}212}$ for inhibition was found to be 2 μM, with complete inhibition at 50 μM. The decrease in secretion induced by myr-syn$A_{233\text{-}245}$ was slightly lower with an IC_{50} of 4 μM. These results agree with previous experiments in permeabilized β cells in which synB showed also a lower IC_{50} value for the inhibition of calcium-induced insulin release(9).

To ensure the specifity of such effect we incubated β cells with control peptides having the same sequence but without the myristoylated chain and in other hand we also used myristoylated peptides having the same composition but in random sequence. Table 1 shows the effect of these peptides at 300 μM final concentration. Only the myristoylated syntaxin-derived peptides displayed a clear inhibitory effect (*$p < 0.0002$) reducing almost 100% glucose-induced secretion whereas control peptides did not show any significative effect. These results allow us to hypothesized that a significa-

TABLE 1

Specificity of the Effect of the Myristoylated Syntaxin-Related Peptides on Glucose-Induced Insulin Release

Agents added during incubation	IRI (pg/1000 cells/10 min)		
	Mean ± SEM	*n*	2*p*
3 mM glucose	183.56 ± 25.03	4	<0.0002
22 mM glucose	803.09 ± 46.08	4	
sA	769.81 ± 46.16	4	NS
msA	272.82 ± 31.98	4	<0.0002
cA	843.81 ± 63.08	4	NS
mcA	871.59 ± 77.28	4	NS
sB	755.46 ± 31.21	4	NS
msB	207.82 ± 21.81	4	<0.0002
cB	722.10 ± 69.98	4	NS
mcB	800.43 ± 76.63	4	NS

Note. Pancreatic β cells were incubated at 37°C in 3 mM basal glucose for 2h in the absence or presence of 300 μM of the myristoylated syntaxin-related peptides, myr-syn $A_{233-245}$ (msA) and myr-syn$B_{200-212}$ (msB), the myristoylated control peptides with random sequences myr-con A (mcA) and myr-con B (mcB), the syntaxin-related non-myristoylated peptides syn $A_{233-245}$ (sA) and syn $B_{200-212}$ (sB), as well as the corresponding control with aleatory sequences, conA (cA) and conB (cB). Stimulation with 22 mM glucose was further performed during 30 additional min in the presence or absence of the myristoylated and non-myristoylated peptides. IRI, immunoreactive insulin. Experimental data are expressed as means ± SEM of 4 experiments performed in triplicate, *$p < 0.0002$ when compared with 22 mM glucose-induced secretion (n = 4).

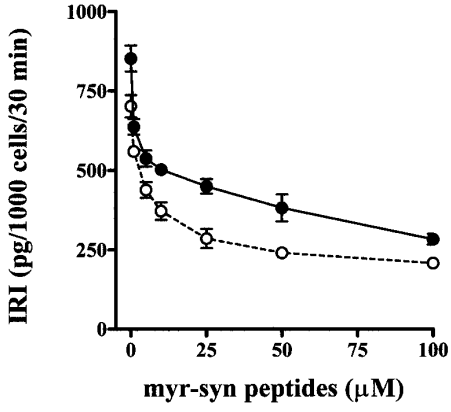

FIG. 2. Dose-dependent effect of syntaxin-related myristoylated peptides (myr-syn $A_{233\text{-}245}$ and myr-syn $B_{200\text{-}212}$) on glucose-induced insulin relase. Pancreatic β cells were incubated at 37°C in the absence or presence of different concentrations of myr-syn $A_{233\text{-}245}$ (●) or myr-syn $B_{200\text{-}212}$ (○) for 2h in 3 mM basal glucose. Stimulation with 22 mM glucose was further performed for an additional period of 30 min in the presence or absence of the different concentrations of the myristoylated peptides assayed. IRI, immunoreactive insulin. Experimental data are expressed as means ± SEM of 3 experiments performed in triplicate.

tive fraction of these myristoylated peptides reaches the cytoplasmatic side of the plasmatic membrane in which the acil chain would be anchored, allowing to the peptide to interact specifically with the secretory machinery in such a way that exocytosis would be decreased supposedly because the competition of myristoylated peptides with endogenous proteins of the secretory complex. It is particularly probable the binding of myr-syn$B_{200\text{-}212}$ with endogenous SNAP25, avoiding its interaction with syntaxin and therefore disrupting exocytosis. Indeed, a recent study has shown that the myristate chain of N-terminal modified peptides interacts hydrophobically with phospholipid membranes maintaining the peptide backbone in an extended conformation which would lie at a distance from the membrane interface according to the nature of their residues (14). In conclusion, this study has demonstrated the possibility of uncoupling secretion in intact cells with syntaxin-related peptides and at the same time, the central role of this protein in nutrient-induced insulin release in β cells. Further studies are intended in order to evaluate the mechanism of accion of these syntaxin-related engineered peptides and their possible application in the regulation of the secretory process in intact cells.

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