Truncation of SNAP-25 reduces the stimulatory action of cAMP on rapid exocytosis in insulin-secreting cells

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SYNAPTOosomal protein of 25 kDa (SNAP-25) is important for Ca2+ dependent fusion of large dense core vesicles (LDCVs) in insulin-secreting cells. Exocytosis is further enhanced by cAMP-increasing agents such as glucagon-like peptide-1 (GLP-1), and this augmentation includes interaction with both PKA and cAMP-GEFII. To investigate the coupling between SNAP-25- and cAMP-dependent stimulation of insulin exocytosis, we have used capacitance measurements, protein-binding assays, and Western blot analysis. In insulin-secreting INS-1 cells overexpressing wild-type SNAP-25 (SNAP-25wt), rapid exocytosis was stimulated more than threefold by cAMP, similar to the situation in nontransfected cells. However, cAMP failed to potentiate rapid exocytosis in INS-1 cells overexpressing a truncated form of SNAP-25 (SNAP-251-197) or Botulinum neurotoxin A (BoNT/A). Close dissection of the exocytotic response revealed that the inability of cAMP to stimulate exocytosis in the presence of a truncated SNAP-25 was confined to the release of primed LDCVs within the readily releasable pool, especially from the immediately releasable pool, whereas cAMP enhanced mobilization of granules from the reserve pool in both SNAP-251-197 (P < 0.01) and SNAP-25wt (P < 0.05) cells. This was supported by hormone release measurements. Augmentation of the immediately releasable pool by cAMP has been suggested to act through the cAMP-GEFII-dependent, PKA-independent pathway. Indeed, we were able to verify an interaction between SNAP-25 with both cAMP-GEFII and RIM2, two proteins involved in the PKA-independent pathway. Thus we hypothesize that SNAP-25 is a necessary partner in the complex mediating cAMP-enhanced rapid exocytosis in insulin-secreting cells.
	synaptosomal protein of 25 kDa; insulin; INS-1; cAMP-GEFII; Epac; capacitance measurements.

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BLOOD GLUCOSE LEVELS ARE REGULATED by the release of insulin from pancreatic β-cells via a process called exocytosis. A crucial mechanism behind the fine-tuned release of insulin is a functioning exocytotic machinery (32, 37). Key players in the exocytotic process where insulin large dense core vesicles (LDCVs) fuse with the plasma membrane are the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins. The SNARE complex comprise the two t-SNARE proteins synaptosomal protein of 25 kDa (SNAP-25) and syntaxin 1A and the v-SNARE protein VAMP2 (9). The amino terminal of SNAP-25 binds to syntaxin 1A and the carboxy-terminal binds to VAMP2 forming the four-helical bundle that brings the vesicular membrane in close contact with the plasma membrane enabling fusion to occur. SNAP-25 consists of 206 amino acids associated to the plasma membrane through palmitoylation of a cysteine-rich central domain (11, 33). The neurotoxins Botulinum neurotoxin A (BoNT/A) and BoNT/E cleave SNAP-25 at Gln197-Arg198 and Arg180-Ile181, respectively, and remove 9 and 26 amino acids in the carboxy terminal (29). This cleavage has been shown to block neuroexocytosis (23, 30, 50) and also insulin secretion (3, 13, 14, 38). SNAP-25 is present in insulin-secreting cells (3, 13, 14, 16, 27, 31, 38, 47, 49) and is suggested to participate as a modulator in several processes apart from the actual fusion event including modulation of Kv2.1 channels (24).

The exocytotic process can be regulated by a diverse variety of inhibitors and stimulators. Incretins, such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), potentiate exocytosis through binding to their specific receptor at the plasma membrane (26). The binding stimulates adenylyl cyclase with a resultant increase in the cAMP concentration and an enhanced exocytotic response (6, 12). Potentiation by cAMP of the exocytotic process involves both PKA-dependent and PKA-independent pathways (7, 34, 36, 41, 44). The latter has been suggested to effect a rapid component of exocytosis and involves the cAMP-binding protein cAMP-GEFII (or Epac; Refs. 7, 34, 41, 44) and the plasma membrane sulphonylurea receptor 1 (SUR1; Refs. 7, 42, 43). Apart from its role in exocytosis cAMP-GEFII is also involved in ion-channel regulation and Ca2+ signaling (18, 19, 22, 25, 45). In addition, cAMP-GEFII interacts with the Rab3a-interacting protein RIM2 (20, 42) involved in neurotransmitter release (39) and insulin exocytosis (20, 21, 42). RIM2 binds to multiple exocytotic proteins, such as Munc-13 (21), L-type Ca2+ channels (5, 42), and SNAP-25 (5), and is suggested to be a scaffolding protein participating in docking and fusion of the LDCVs.

To get further insight into the mechanisms by which SNAP-25 is functionally associated to cAMP-stimulated exocytosis in insulin-secreting cells, we performed depolarization-induced capacitance measurements on INS-1 cells transfected either with BoNT/A or a truncated form of SNAP-25 (SNAP-251-197) combined with binding studies of proteins involved in the cAMP-stimulated, PKA-independent pathway. From these measurements, we found that rapid cAMP-induced exocytosis is impaired in cells overexpressing SNAP-25 truncated at the C terminus and that both full-length and truncated SNAP-25 bind to both cAMP-GEFII and RIM2. Thus these results suggest that SNAP-25 is essential for cAMP-induced rapid exocytosis.
in insulin-secreting cells and that the transducing domain of SNAP-25 on cAMP-mediated exocytosis is at the C terminus of SNAP-25, whereas the major binding domains are toward the N terminus.

**MATERIALS AND METHODS**

**Single cell capacitance measurements.** Rat insulinoma (INS-1; kind gift from C. Wollheim) cells were cultured with RPMI 1640 media containing 10% FCS (wt/vol), 50 μM 2-mercaptoethanol, 100 IU/ml penicillin, and 100 μg/ml streptomycin. INS-1 cells were transiently cotransfected with enhanced green fluorescent protein (EGFP) and a pcDNA3 vector containing wild-type SNAP-25 (SNAP-25WT; Refs. 14, 17), truncated SNAP-25 (SNAP-251-197; Refs. 14, 17), or BoNT/A (13, 14) for 48 h using Effectene (Qiagen Nordic, Solna, Sweden).

Whole cell currents and exocytosis were recorded using an EPC-9 patch-clamp amplifier (HEKA electronics; http://www.heka.com) and the software Pulse (ver 8.50). Measurements were performed on EGFP-positive INS-1 cells and non-EGFP-positive (nontransfected) cells in the same dish. Exocytosis was recorded as changes in membrane capacitance using the standard whole-cell configuration of the patch-clamp technique. The extracellular medium contained the following (in mM): 118 NaCl, 20 tetra-ethyl-ammonium chloride (to block voltage-gated K⁺ currents), 5.6 KCl, 2.6 CaCl₂, 1.2 MgCl₂, 5 glucose, and 5 HEPES (pH 7.4 using NaOH). The pipette solution consisted of the following (in mM): 125 Cs-glutamate, 10 NaCl, 10 CsCl, 1 MgCl₂, 0.05 EGTA, 3 Mg-ATP, and 10 HEPES (pH 7.15 with CsOH). cAMP (0.1 mM) was added as indicated in the text. Patch electrodes were pulled from borosilicate capillaries, coated with Sylgard (Dow Corning Midland), and fire polished. The pipette resistance was 3–7 MΩ when the pipettes were filled with the intracellular solution. Exocytosis was detected as changes in membrane capacitance using the software-based lock-in application (which adds a sine wave of 500–1000 Hz to the holding potential) of the amplifier. Exocytosis was elicited by single depolarizations from −70 to 0 mV of increasing duration (5–450 ms) or by a train of ten 500-ms voltage-clamp depolarizations from −70 to 0 mV applied at 1 Hz. All experiments were conducted at 32–34°C.

**Confocal immunofluorescence microscopy.** INS-1 or MIN6 (kind gift from S. Seino, Kobe University, Japan) cells were grown at 37°C in 5% CO₂ in DMEM (Sigma-Aldrich) containing 25 mM glucose supplemented with 10% FBS and 100 U/ml penicillin/streptomycin. Plasmid cDNA (1–5 μg) encoding BoNT/A was cotransfected with 0.2 μg pcDNA3 vector containing SNAP-25WT; (14, 17), truncated SNAP-25 (SNAP-251-197; Refs. 14, 17), or empty pcDNA3 and 0.2 g human growth hormone (hGH) expressing vector (48) using Effecene transfection kit (Qiagen). After 24 h, 1 ml RPMI 1640 medium was added. After 48 h, the cells were washed (500 μl) and preincubated (300 μl) for 30 min at 37°C in the following (in mM): 20 HEPES, 128 NaCl, 5 KCl, 1 MgCl₂, 2.7 CaCl₂, 0.1% BSA, and 3 glucose (pH 7.4). The cells were then incubated (300 μl) for 60 min in either the same buffer containing either 3 mM glucose, 20 mM glucose, or 20 mM glucose in combination with 0.1 mM IBMX and 10 μM forskolin (pH 7.4). The secretion samples (200 μl) were centrifuged to remove the cell debris. The cells were harvested in 200 μl PBS and 0.1% Triton X-100, sonicated, and centrifuged to remove cell debris. Analysis of hGH content in secretion samples and cell homogenates was made with hGH ELISA (Roche Diagnostics, Stockholm, Sweden). Data are presented as the ratio of secreted hGH to total hGH content/well to compensate for variations in transfection efficiency.

**Statistics.** Data are presented as means ± SEM of indicated experiments (n). Statistical significances were assessed using Student’s t-test or ANOVA.

**RESULTS**

SNAP-25 at its N-terminal domain binds to cAMP-GEFII and RIM2. SNAP-25 has in several studies (13, 31, 49) been shown to have an important role in the exocytotic process of insulin-secreting cells. Here we were specifically interested in investigating the role of SNAP-25 in cAMP-stimulated exocytosis. We first examined whether SNAP-25WT and SNAP-251-197 bind to the major exocytotic proteins acted on by cAMP, which are cAMP-GEFII and its downstream protein.
RIM2 (20, 42). To examine for the direct interactions of these proteins, cAMP-GEFII or RIM2 were expressed in TSA cells, which endogenously express a minimal amount of cAMP-GEFII and no RIM2. The TSA cells were then subjected to pull-down studies of the SNAP-25 proteins bound to glutathione agarose beads (Fig. 1). Surprisingly, SNAP-251-197 pulled down more cAMP-GEFII and RIM2 than the SNAP-25 WT protein, while GST control did not bind either protein (Fig. 1A). It was ascertained that equal amounts of the SNAP-25 proteins and GST were used for the pull-down study (Fig. 1B).

Full-length SNAP-25 (SNAP25 WT) overexpression in INS-1 cells enhances cAMP-stimulated exocytosis. The exocytotic process in insulin-secreting cells can be subdivided into the release of LDCVs residing in different pools before release (1, 37). LDCVs within a reserve pool (RP) need to be mobilized and undergo Ca2⁺/H11001- and ATP-dependent priming before the release, whereas LDCVs within the readily releasable pool (RRP) have already undergone the priming process and are released immediately upon Ca2⁺ increase. A subpopulation of RRP, the immediately releasable pool (IRP), is closely associated with the L-type Ca2⁺/H11001 channels (2).

To investigate the possible involvement of SNAP-25 in cAMP-dependent enhancement of IRP in detail, experiments were performed in single INS-1 cells expressing SNAP-25 variants (13, 14, 17). The limitation of the transfection rate was overcome by coexpressing the construct together with EGFP, and thereby the transfection rate was irrelevant when the capacitance measurements were performed. Western blot analysis was performed to verify the presence of SNAP-25 in untransfected INS-1 cells and the increased expression of SNAP-25 (~4-fold) when INS-1 cells were transfected with SNAP-25 WT (Fig. 2A).

Exocytosis was elicited by voltage-clamp depolarizations from −70 to 0 mV with increasing duration from 5 to 450 ms (Fig. 2, B-D). In cells overexpressing SNAP-25 WT, a 50-ms depolarization evoked a capacitance increase of 7 ± 2 fF (n = 6) under control conditions. This was increased to 44 ± 10 fF (P < 0.05; n = 13) in the presence of 0.1 mM cAMP. A similar sixfold (P < 0.01) increase could be observed when exocytosis was elicited by a 250-ms depolarization. IRP and the time constant of the capacitance increase (τ) were estimated by fitting an equation to the data points (Fig. 2D). This equation describes the kinetics when the LDCVs are moving from IRP into a fused state (49). The results of the fitting from several series under different conditions are presented in Table 1. In the above experimental series, the size of IRP was increased from 51 ± 12 fF (n = 6) in the control cells to 182 ± 18 fF (P < 0.001; n = 13) in the presence of cAMP, whereas τ did not change. We also made the observation that the results from INS-1 cells overexpressing SNAP-25 WT were similar to nontransfected cells (Table 1), which is similar to insulin secretion measurements in HIT T15 cells already published (14). The increases in membrane capacitance measured in SNAP-25 WT cells and nontransfected cells are also similar to earlier published data on INS-1 cells overexpressing EGFP alone (15).

![Fig. 1. Wild-type synaptosomal protein of 25 kDa (SNAP-25 WT) and truncated SNAP-25 (SNAP-251-197) bind cAMP-GEFII and RIM2 proteins. Binding assays were performed using GST (negative control), GST-SNAP-25, and GST-SNAP-251-197 (all bound to glutathione agarose beads, 400 pmol protein each) to pull down overexpressed cAMP-GEFII-WT or RIM2 from TSA cell lysate extract. A, top panel: representative Western blots of the precipitated cAMP-GEFII-WT (left top, lanes 1–3) and RIM2 proteins (right top, lanes 5–7). TSA cell lysate extract-1 (containing overexpressed cAMP-GEFII-WT protein, lane 4) and extract-2 (containing overexpressed RIM2 protein, lane 8) were used as positive controls. Bottom: summary of quantitative densitometric scanning of blots from 3 separate experiments. Values are means ± SE are expressed as a percentage of the maximum. B, representative Ponceau S staining of the blot to show the equal amount of GST proteins loaded.](http://ajpendo.physiology.org/doi/abs/10.1152/ajpendo.00014.2009)
C-terminal truncated SNAP-25 renders INS-1 cells insensitive to cAMP potentiation. To investigate a possible interaction between SNAP-25 and cAMP, we next overexpressed the C-terminal truncated form of SNAP-25 (SNAP-25<sub>1-197</sub>). The overexpression was verified using Western blot analysis (Fig. 2A). Exocytosis was evoked as described in Full-length SNAP-25 (SNAP<sub>25WT</sub>) overexpression in INS-1 cells enhances C-terminal truncated SNAP-25 renders INS-1 cells insensitive to cAMP potentiation. To investigate a possible interaction between SNAP-25 and cAMP, we next overexpressed the C-terminal truncated form of SNAP-25 (SNAP-25<sub>1-197</sub>). The overexpression was verified using Western blot analysis (Fig. 2A). Exocytosis was evoked as described in Full-length SNAP-25 (SNAP<sub>25WT</sub>) overexpression in INS-1 cells enhances C-terminal truncated SNAP-25 renders INS-1 cells insensitive to cAMP potentiation. To investigate a possible interaction between SNAP-25 and cAMP, we next overexpressed the C-terminal truncated form of SNAP-25 (SNAP-25<sub>1-197</sub>). The overexpression was verified using Western blot analysis (Fig. 2A). Exocytosis was evoked as described in Full-length SNAP-25 (SNAP<sub>25WT</sub>) overexpression in INS-1 cells enhances C-terminal truncated SNAP-25 renders INS-1 cells insensitive to cAMP potentiation. To investigate a possible interaction between SNAP-25 and cAMP, we next overexpressed the C-terminal truncated form of SNAP-25 (SNAP-25<sub>1-197</sub>). The overexpression was verified using Western blot analysis (Fig. 2A). Exocytosis was evoked as described in Full-length SNAP-25 (SNAP<sub>25WT</sub>) overexpression in INS-1 cells enhances C-terminal truncated SNAP-25 renders INS-1 cells insensitive to cAMP potentiation. To investigate a possible interaction between SNAP-25 and cAMP, we next overexpressed the C-terminal truncated form of SNAP-25 (SNAP-25<sub>1-197</sub>). The overexpression was verified using Western blot analysis (Fig. 2A). Exocytosis was evoked as described in Full-length SNAP-25 (SNAP<sub>25WT</sub>) overexpression in INS-1 cells enhances C-terminal truncated SNAP-25 renders INS-1 cells insensitive to cAMP potentiation. To investigate a possible interaction between SNAP-25 and cAMP, we next overexpressed the C-terminal truncated form of SNAP-25 (SNAP-25<sub>1-197</sub>). The overexpression was verified using Western blot analysis (Fig. 2A). Exocytosis was evoked as described in Full-length SNAP-25 (SNAP<sub>25WT</sub>) overexpression in INS-1 cells enhances C-terminal truncated SNAP-25 renders INS-1 cells insensitive to cAMP potentiation. To investigate a possible interaction between SNAP-25 and cAMP, we next overexpressed the C-terminal truncated form of SNAP-25 (SNAP-25<sub>1-197</sub>). The overexpression was verified using Western blot analysis (Fig. 2A). Exocytosis was evoked as described in Full-length SNAP-25 (SNAP<sub>25WT</sub>) overexpression in INS-1 cells enhances 

**Table 1. Summary of the effects of cAMP and truncation of SNAP-25 on the kinetics of exocytosis in the INS-1 cells**

<table>
<thead>
<tr>
<th>Overexpression</th>
<th>Condition</th>
<th>IRP, fF</th>
<th>τ, ms</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>no cAMP</td>
<td>89 ± 12</td>
<td>20 ± 5</td>
<td>30</td>
</tr>
<tr>
<td>None</td>
<td>0.1 mM cAMP</td>
<td>197 ± 39</td>
<td>18 ± 5</td>
<td>11</td>
</tr>
<tr>
<td>SNAP-25&lt;sub&gt;WT&lt;/sub&gt;</td>
<td>no cAMP</td>
<td>52 ± 12</td>
<td>28 ± 15</td>
<td>6</td>
</tr>
<tr>
<td>SNAP-25&lt;sub&gt;WT&lt;/sub&gt;</td>
<td>0.1 mM cAMP</td>
<td>182 ± 18</td>
<td>20 ± 7</td>
<td>13</td>
</tr>
<tr>
<td>SNAP-25&lt;sub&gt;1-197&lt;/sub&gt;</td>
<td>0.1 mM cAMP</td>
<td>86 ± 20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20 ± 8</td>
<td>9</td>
</tr>
<tr>
<td>BoNT/A</td>
<td>no cAMP</td>
<td>60 ± 8</td>
<td>10 ± 4</td>
<td>7</td>
</tr>
<tr>
<td>BoNT/A</td>
<td>0.1 mM cAMP</td>
<td>100 ± 14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25 ± 6</td>
<td>29</td>
</tr>
</tbody>
</table>

Values of IRP and τ were derived from approximating an equation, describing exocytosis of IRP granules (49), to the individual data points in Figs. 2, 3 and 5. Data are mean values ± SE of indicated number of cells investigated (n). *P < 0.001 vs. line 1, *P < 0.001 vs. line 3, 3P < 0.001 vs. line 2, 4P < 0.01 vs. line 4, 5P < 0.01 vs. line 2. Statistical significance refers to comparison within the same column.

cAMP-stimulated exocytosis. Under these conditions, exocytosis was not potentiated to the same extent by cAMP (Fig. 3) as in the cells overexpressing full-length SNAP-25. Specifically, the increase in membrane capacitance in SNAP-25<sub>1-197</sub> cells evoked by the 250-ms depolarization was 35 ± 2 fF (n = 26) and 73 ± 22 fF (n = 9; P < 0.05) in the absence and presence of cAMP, respectively. Although cAMP significantly enhanced exocytosis in cells overexpressing SNAP-25<sub>1-197</sub>, the potentiating effect was only 30% compared with SNAP-25<sub>WT</sub> cells.

The data from these experiments are summarized in Fig. 3C and were used to estimate IRP and the time constant τ (Table 1). The estimated size of IRP was 53 ± 8 fF (n = 26) and 86 ± 20 fF (n = 9) in the absence and presence of cAMP, respectively, suggesting that the capacity by which cAMP is able to increase IRP is reduced by ~70% (P < 0.01; n = 9–13) in SNAP-25<sub>1-197</sub> cells compared with SNAP-25<sub>WT</sub> cells.

BoNT/A treatment of INS-1 results in inability to enhance exocytosis with cAMP. We were next interested in investigating if BoNT/A treatment might have the same effect as exog- enously expressed SNAP-25<sub>1-197</sub>, since BoNT/A cleavage of endogenous SNAP-25 would generate N-terminal SNAP-25<sub>25-197</sub> and C-terminal 8 amino acid fragments. INS-1 cells were therefore transfected with a vector containing BoNT/A. Before the electrophysiological investigations, it was con- firmed that BoNT/A transfection completely cleaved and de- leted the endogenous plasma membrane-bound SNAP-25. We first examined this by confocal microscopy of INS-1 cells 24 and 48 h of culture after the BoNT/A transfection (Fig. 4A). SNAP-25 is present in the plasma membrane of untreated INS-1 cells. GFP-expressing cells would be expected to contain the coexpressed BoNT/A, as the INS-1 cells are known to
pick up multiple plasmids. Of note, GFP-expressing cells contained an abundance of SNAP-25-immunoreactive proteins in the cytosol, which overlapped with the cytosolic GFP staining. In non-GFP-containing cells, SNAP-25 remained on the plasma membrane.

To determine the identity of the cytosolic SNAP-25-immunoreactive protein resulting from BoNT/A transfection observed in the confocal microscopy study, the BoNT/A-transfected cell lysates were immunoblotted to detect SNAP-25 cleavage products (Fig. 4B). BoNT/A cleavage of the 206 amino acid SNAP-25 protein at residues 197–198 was expected to generate a N-terminal SNAP-25_1-197 domain protein, which is 1 kDa smaller than full-length SNAP-25 (29). Indeed, BoNT/A transfection caused a slight reduction in the level of parent SNAP-25 (indicated by arrowheads) and the corresponding appearance of a 1 kDa smaller SNAP-25 protein (indicated by arrows), which was more prominent at 48 h of culture. INS-1 cells, although an excellent model to examine exocytosis by capacitance measurement, exhibit low transfection efficiency (only ~20–30%). We therefore employed MIN6 cells, which can be transfected with much higher efficiency (~60%). Indeed, in these cells native SNAP-25 was more effectively cleaved (shown more clearly on the shorter 2-s film exposure), generating a more abundant SNAP-25_1-197 domain fragment (shown more clearly on the 4-s film exposure). These results indicate that the expressed BoNT/A progressively increased cleavage of SNAP-25 but the resulting N-terminal SNAP-25_1-197 fragment did not undergo substantial further degradation but was retained intact in the cytosol.

Taken together, these results suggest that the effects of BoNT/A on insulin exocytosis might not be due to complete destruction of SNAP-25 per se but could be attributed to the cleaved N-terminal SNAP-25_1-197 fragment that can be retained for long periods. If this is the case, then this would mimic BoNT/A poisoning of neurons (8, 35) and neuroendocrine cells (30), where the N-terminal SNAP-25 fragment can be retained for a period of months.

Finally, cAMP stimulation of BoNT/A-transfected cells was investigated by capacitance measurements (Fig. 5). As in the cells overexpressing SNAP-25_1-197, cAMP failed to enhance exocytosis to the same amount as in the SNAP-25WT cells, and the capacitance increase due to a 250-ms depolarization was 47 ± 8 (n = 7) and 69 ± 12 (n = 29) in the absence and presence of cAMP, respectively. In concomitance, the size of IRP and the time constant τ in the BoNT/A cells was not changed in the presence of cAMP compared with control (Table 1). Thus, these experiments taken along with the previous study suggest that the cAMP transduction domain of SNAP-25 is at the C terminus (amino acid 198–206) of SNAP-25.

Mobilization is reduced in INS-1 cells overexpressing truncated SNAP-25. The protocols used above only measures rapid exocytosis of IRP granules but does not give further information about RRP and LDCVs mobilized/translocated from RP. To investigate the latter properties, INS-1 cells transfected with SNAP-25WT (Fig. 6A) or SNAP-25_1-197 were subjected to a train of 500-ms depolarizations from −70 to 0 mV. The total capacitance increase in the SNAP-25WT cells was 155 ± 56 fF (n = 16; P < 0.05) in the absence and presence of cAMP, respectively. The same values for the SNAP1-197 cells were 140 ± 24 fF (n = 21) and 295 ± 56 fF (n = 11; P < 0.01). Dissecting the trains further showed that the estimated maximum size of RRP (Fig. 6C) achieved from the sum of the two first depolarizations of the train (10) was significantly increased by cAMP in both the SNAP-25WT and the SNAP-25_1-197 cells. However, the increase of RRP was
significantly lower in the SNAP-25_{1-197} cells compared with the SNAP-25_{WT} cells. In contrast, mobilization of LDCVs from the RP measured as the increases in membrane capacitance during the latter depolarizations (depol 3–10; Fig. 6D) was remarkably similar in the SNAP-25_{WT}- and SNAP-25_{1-197}-expressing cells, respectively. These results indicate that SNAP-25 influences the actions of cAMP on IRP/RRP rather than the cAMP effect on mobilization of LDCVs from RP.

We were also interested in investigating glucose-stimulated and cAMP-potentiated insulin secretion in INS-1 cells overexpressing SNAP-25_{WT} and SNAP-25_{1-197}, respectively. To be able to measure insulin secretion only from the transfected cells, we made use of a GH assay previously described (15, 46). In this assay, the SNAP-25 constructs are coexpressed with GH resulting in that only the transfected cells will release GH, which is localized and secreted together with insulin. As a control, we used cells transfected with empty vector (cDNA3) together with GH. INS-1 cells were incubated for 60 min in either 3 mM glucose (basal), 20 mM glucose, or in a solution containing 20 mM glucose in combination with 0.1 mM IBMX and 10 μM forskolin. The latter was used to get maximal glucose-stimulated and cAMP-enhanced secretion. We observed no significant difference within the groups under basal conditions (Fig. 6E). A stimulating glucose concentration (20 mM) had no effect alone, which was not unexpected since INS-1 cells often are not as glucose responsive as primary cells (40). In the presence of cAMP-increasing agents, secretion was amplified three- to fourfold. The cAMP-elevating effect was similar in SNAP-25_{WT} and SNAP-25_{1-197} cells, which agrees with the results from the above capacitance measurements where mobilization is stimulated using a train of depolarizations.

**DISCUSSION**

Insulin secretion is dependent on functional SNARE exocytic machinery where SNAP-25 is an important component.
Sufficient insulin secretion relies on modulators that have the ability to potentiate the exocytotic response. Such mediators are the hormones GLP-1 and GIP (6, 12) as well as the adenylate cyclase activator forskolin (7, 36) that increases intracellular cAMP concentration and thereby enhances the exocytotic process by PKA-independent and PKA-dependent processes (7, 34, 36, 41). The PKA-independent pathway involves cAMP-GEFII and the downstream protein RIM2 (20, 42, 43). In our aim to reveal the mechanism behind exocytosis in insulin-secreting cells, we have attempted to survey the link between SNAP-25- and cAMP-dependent pathways. We demonstrate a binding to cAMP-GEFII with SNAP-25WT as well as with a C-terminal truncated form of SNAP-25 (SNAP-251-197; Fig. 1). Binding could also be confirmed with RIM2. This indicates that the major binding domain(s) are situated toward the N terminus of SNAP-25, since the truncated SNAP-25 protein also has a strong binding to cAMP-GEFII and RIM2. The observed binding implies that the cAMP-dependent, PKA-independent pathway involves a stimulatory action through SNAP-25. Indeed, when investigating exocytosis using capacitance measurements in INS-1 cells overexpressing SNAP-251-197 (Fig. 3) or BoNT/A (Fig. 5), cAMP-enhanced exocytosis was reduced.

We further performed a detailed analysis of the exocytotic response in relation to SNAP-25 and cAMP. Investigations of rapid exocytosis revealed that IRP in the INS-1 cells is increased more than threefold by cAMP in the SNAP-25WT cells. This is similar to the response observed in the nontransfected INS-1 cells, suggesting that endogenous SNAP-25 is already at saturating capacity to potentiate exocytosis through cAMP. This conclusion is in agreement with our previous results on insulin secretion in HIT T15 cells (14). Our capacitance measurements on INS-1 cells are also in general agreement with our previous study (13). However, in the current study, we have examined more in-depth the precise kinetics of the secretory granule pools and the underlying structure-functional analysis of the interacting exocytotic proteins. Both SNAP-251-197 and BoNT/A treatment caused a reduction of the IRP compared with SNAP-25WT, indicating that the cAMP-dependent rapid component of exocytosis is most likely mediated through SNAP-25. This is consistent with our previous study (13, 49) showing that perturbation of SNAP-25 abrogated rapid exocytosis. The rapid component of exocytosis is missing in mice lacking cAMP-GEFII (Epac2Δ/Δo; Ref. 44) and has been suggested to be enhanced through the cAMP-GEFII-dependent pathway (7). Consistently, we here and others (5, 39) showed that SNAP-25 binds to cAMP-GEFII and RIM2. RIM proteins could further bind to the L-type Ca²⁺ channel (5, 39, 41–42), and the L-type Ca²⁺ channel has been demonstrated to be closely associated with SNAP-25 (49). Taken together, this reinforces the fundamental role of SNAP-25 in the release of LDVs within IRP/RRP that involves a unique “excitosome” architecture comprised of cAMP-GEFII, RIM2, and the L-type Ca²⁺ channels (42).

Interestingly, full-length SNAP-25 is needed for maximal cAMP-potentiated exocytosis, even though SNAP-251-197 binds strongly to both cAMP-GEFII and RIM2. This suggests that the C terminus of the full-length SNAP-25 is the major putative domain accounting for transducing the cAMP-potentiating signal on rapid exocytosis. It might be puzzling that although SNAP-251-197 binds strongly to RIM2 and cAMP-GEFII, it is unable to transduce the cAMP-enhancing signal on rapid exocytosis. The most likely reason would be that the N- and C-terminal parts of SNAP-25 have different roles in the multi-protein complex. We therefore suggest that the SNAP-25 N-terminal domain plays a more important role in stabilizing the complex formed with cAMP-GEFII and RIM2, whereas the C-terminal part is more responsible for transducing the augmented effect. The stabilizing effect through the N-terminal binding could certainly induce conformational changes to the multi-protein complex that might be...
required for the SNAP-25 C terminus to then transduce the potentiating signal for the release of IRP/RRP.

Surprisingly, the cAMP-stimulated mobilization of LDCVs in the SNAP-25\(^{1-197}\) cells is not significantly different from SNAP-25\(^{WT}\) cells (Fig. 6). However, these data are supported by the hormone secretion measurements (Fig. 6E), which demonstrate that glucose-stimulated and cAMP-potentiated secretion is similar in SNAP-25\(^{WT}\)- and SNAP-25\(^{1-197}\)-expressing cells when measured over 60 min. Mobilization has earlier been suggested to be mainly PKA dependent (36), and it has also been shown that PKA phosphorylates SNAP-25 at Thr-138 (28). That PKA phosphorylation of SNAP-25 remains possible in the truncated SNAP-25 (since Thr-138 is not removed) would explain why mobilization can still be elevated by cAMP. This novel and intriguing finding raises the possibility that different conformations of the SNAP-25/RIM2/cAMP-GEFII complex could have distinct effects on granule pools, with the SNAP-25 N-terminal complex showing a preference for transducing cAMP-potentiated granule mobilization and the SNAP-25 C-terminal complex preferring to mediate cAMP amplification and release of the IRP/RRP. Nonetheless, the SNAP-25 N-terminal complex is still functional, albeit at much reduced efficacy, and thus accounts for the partial rescue by cAMP.

Based on the present work, we propose that SNAP-25 is essential in cAMP-enhanced insulin secretion and that this effect is most likely mediated through the cAMP-GEFII/RIM2 pathway. These insights are of particular relevance to type 2 diabetes where the pancreatic islet expression of SNAP-25 (32) is diminished and where first phase insulin secretion is reduced to near absence occurring before a subsequent reduced efficiency of first-phase release (4). Since IRP contributes more to first phase insulin release (1), our findings would suggest that the reduced \(\beta\)-cell SNAP-25 C-terminal transduction pathway might be more vulnerable in diabetes than the SNAP-25 N-terminal pathway, with the latter influencing insulin granule pool mobilization that contributes more to second phase insulin secretion. Our observations therefore suggest a novel mechanistic insight into islet \(\beta\)-cell insulin secretory deficiency in diabetes and should be considered in the design of new pharmacological drugs elevating the cAMP levels that act to potentiate the biphasic insulin secretory response.

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