Phosphorylation of SNAP-25 on serine-187 is induced by secretagogues in insulin-secreting cells, but is not correlated with insulin secretion

Carmen GONELLE-GISPERT*, Maria COSTA*, Masami TAKAHASHI†, Karin SADOUL‡ and Philippe HALBAN*

*Laboratoires de Recherche Louis Jeantet, Centre Médical Universitaire, rue Michel Servet, CH-1211 Geneva 4, Switzerland, †Project Research Center, MitsubishiKasei Institute of Life Sciences, Tokyo 194-8511, Japan, and ‡LEDAC, Institut Albert Bonniot, Faculté de Médecine, Domaine de la Merci, 38706 La Tronche Cedex, France

INTRODUCTION

The tSNARE (the target-membrane soluble NSF-attachment protein receptor, where NSF is N-ethylmaleimide-sensitive fusion protein) synaptosomal-associated protein of 25 kDa (SNAP-25) is implicated in regulated insulin secretion. In pheochromocytoma PC12 cells, SNAP-25 is phosphorylated at Ser187, which lies in a region that is important for its function. The aims of the present study were to determine whether SNAP-25 is phosphorylated at Ser187 in insulin-secreting cells and, if so, whether this is important for regulated insulin secretion. The major findings are: (i) SNAP-25 is rapidly and reversibly phosphorylated on Ser187 in both rat insulinoma INS-1 cells and rat islets in response to the phorbol ester, PMA; (ii) less than 35% of SNAP-25 in INS-1 cells is phosphorylated in response to PMA, and phosphorylation is limited to plasma-membrane-associated SNAP-25; (iii) both SNAP-25 isoforms (a and b) are phosphorylated, with 1.8-fold greater phosphorylation for SNAP-25b in response to PMA; (iv) in rat islets, Ser187 phosphorylation is stimulated by glucose or carbachol, albeit to a lesser extent than by PMA, but not by cAMP; (v) insulin secretion from botulinum neurotoxin E-treated hamster insulinoma tumour (HIT) cells, transfected with toxin-resistant Ser187→Ala or Ser187→Asp mutant SNAP-25, was similar to that of wild-type HIT cells. Furthermore, in rat islets no correlation was found between the extent of SNAP-25 phosphorylation at Ser187 in response to secretagogues and stimulation of insulin release; (vi) use of protein kinase C (PKC) inhibitors suggests that glucose stimulates SNAP-25 phosphorylation via conventional and non-conventional PKC isoforms. In summary, although SNAP-25 phosphorylation at Ser187 occurs in insulin-secreting cells and is mediated by PKC, it does not appear to play a major role in regulated insulin secretion.

Key words: exocytosis, islets, phosphorylation, protein kinase C, soluble NSF (N-ethylmaleimide-sensitive fusion protein)-attachment protein receptor (SNARE).

Abbreviations used: BIS, bisindolylmaleimide I; BoNT, botulinum neurotoxin; CaMKII, Ca2+/calmodulin-dependent protein kinase II; CasKII, casein kinase II; Ch, carbachol; DAG, diacylglycerol; DPBS, Dulbecco’s PBS; FCS, foetal calf serum; GFP, green fluorescent protein; HIT, hamster insulinoma tumour; IBMX, isobutylmethylxanthine; I.P., immune precipitate; KRB, Krebs–Ringer bicarbonate buffer; NGF, nerve growth factor; NSF, N-ethylmaleimide-sensitive fusion protein; PDA, phorbol 12,13-diacetate; PMA, protein kinase A; PKC, protein kinase C; SNAP-25, synaptosomal-associated protein of 25 kDa; (v)SNARE, (target/membrane) soluble NSF-attachment protein receptor; TR, toxin resistant; TRITC, tetramethylrhodamine β-isothiocyanate; VAMP, vesicle-associated membrane protein; wt, wild-type.

1 To whom correspondence should be addressed (e-mail carmen.gonelle@medecine.unige.ch).
ation is induced by phorbol 12,13-dibutyrate or long-term poten-
tiation, which is known to be important in the mechanism
implicated in memorization and learning [19].

In PC12 cells, SNAP-25 phosphorylation at the PKC consensus
site on Ser187 has been shown to be inducible by the phorbol ester
PMA, or following nerve growth factor (NGF) treatment [20]. It
was reported further that phosphorylation of SNAP-25 induces
alterations of its intracellular distribution [21]. The purpose of
the present study was to examine SNAP-25 phosphorylation at
Ser187 in insulin-secreting cells and to determine whether there is
any correlation between phosphorylation and insulin secretion.

EXPERIMENTAL

Materials

Culture medium and serum was from Gibco BRL (Life Tech-
nologies AG, Basel, Switzerland) and all chemicals used were
from Sigma-Fluka (Buchs, Switzerland) unless otherwise stated.
The cDNA coding for human SNAP-25b [22] was cloned into the
pcDNA3 vector (Invitrogen, Groningen, The Netherlands).
Cloning of myc-tagged SNAP-25a and -25b constructs [23] and
green fluorescent protein (GFP)–SNAP-25 constructs, as well as
mutagenesis of the GFP–SNAP-25 cysteine mutants are des-
cribed elsewhere [24]. The toxin resistant (TR) GFP–SNAP-
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cribed elsewhere [24]. The toxin resistant (TR) GFP–SNAP-
25bTR was used as a template for both mutations, using
Clone and -25b constructs [23] and
pcDNA3 vector (Invitrogen, Groningen, The Netherlands).
the QuickChange® Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.), according to
the manufacturer's instructions. The construct pcDNA3–GFP–
SNAP-25bTR was used as a template for both mutations, using
the following primers: Ser187 → Ala mutation, 5'-GGGAGAGG-
GCTGACCACAACAAACACAG-3' and 3'-GATCTTCGGAG-
CTGGTGTGTATGGTC-5'; Ser187 → Asp mutation, 5'-GGGA-
GAGCTGACTCCAACACACACAG-3' and 3'-CTCCTTCTCCGA-
CTGGTGTGTATGGTC-5'. The monoclonal antibody that recog-
nized total SNAP-25 (SMI81) was obtained from
Sternberger Monoclonals Inc. (Baltimore, MD, U.S.A.). The
polyclonal antibody against SNAP-25, 116b, was a gift from Dr
Stefan Catsicas. Generation of the polyclonal antibody raised
against a phospho-Ser187 peptide equivalent to SNAP-25 residues
182–192 has been described previously [21]. The secondary
antibodies tetramethylrhodamine β-isothiocyanate (TRITC)–
anti-mouse and FITC–anti-rabbit were from Jackson Immuno-
research Laboratories (West Grove, PA, U.S.A.) and Sigma re-
spectively. The PKC inhibitors Gö 6976 and bisindoylmaleimide
1 (BIS) were purchased from Calbiochem (San Diego, CA,
U.S.A.).

Cell lines

Rat insulinoma INS-1 cells [25] were cultured in RPMI 1640
containing 10% foetal calf serum (FCS) and supplemented with
10 mM Hepes, 2 mM glutamine, 1 mM sodium pyruvate and
0.05 mM 2-mercaptoethanol. Hamster insulinoma tumour (HIT)
cells were cultured [26] in RPMI 1640, 10% FCS, 32.5 μM
glutathione, 0.1 μM selenious acid.

Transfection of INS-1 and HIT cells

INS-1 cells were seeded at a density of 0.5 × 10⁶ cells/Petri dish
of 10 cm². After 2 days, cells were transfected using 4 μl of
Effectene transfection reagent (Qiagen, Basel, Switzerland) for
0.4 μg of total DNA/dish, in a final volume of 0.6 ml of complete
culture medium. Transfection medium was changed after over-
night incubation and transfected cells were used for further
experiments 2 days after transfection. In order to obtain equal
transfection levels of myc–SNAP-25a and myc–SNAP-25b, twice
as much DNA of the former was used (0.2 μg of pcDNA3–
myc–SNAP-25b and 0.2 μg of the empty pcDNA3 vector, in
contrast with 0.4 μg of pcDNA3–myc–SNAP-25a alone). For
the transfection assay, HIT cells were seeded at a density of
4.5 × 10⁵ cells/well in 24-well plates. Transfection was performed
using 1 μl of Transfectam (Promega) for 5 μg of total DNA/well
in a total volume of 0.4 ml of culture medium without FCS for
4-6 h. The following quantities of DNA were used for the
transfection assays: 2.5 μg of pCMV human pro-insulin plus 0.2 μg
from either pcDNA3–GFP–SNAP-25bTRwt (wild-type) or
Ser187 → Ala or Ser187 → Asp mutants plus 2.3 μg of pcDNA3.
For control conditions, 2.5 μg of pcMV human pro-insulin plus
2.5 μg of pcDNA3 were used. The transfection efficiency was
approx. 10% in all experiments.

Immunofluorescence

At 2 days before treatment, INS-1 cells were seeded at
0.5 × 10⁶ cells/dish. Cells were incubated in culture medium in
the presence or absence of 1 μM PMA for 1 h and afterwards
fixed and permeabilized with ice-cold methanol for 2 min. Cells
were washed twice with Dulbecco's PBS (DPBS) and blocked for
20 min with DPBS containing 5% BSA. Before incubation with
SMI81, diluted in PBS containing 1% BSA at 1:400, together
with the polyclonal antibody SN25Pi, diluted at 1:500, for 2 h at
room temperature (22 °C). Cells were again washed twice with
DPBS and incubated with the secondary antibodies for 1 h at
room temperature using TRITC–anti-mouse diluted at 1:400
and FITC–anti-rabbit (1:100). Fluorescence images were taken
using an AxioCam camera adapted to a fluorescence microscope
(Zeiss).

SDS/PAGE and Western blotting

Proteins were resolved on 10% polyacrylamide gels under
reducing or non-reducing conditions according to Laemmli [27].
After SDS/PAGE, proteins were transferred on nitrocellulose
membranes and total SNAP-25 was detected using SMI81
(diluted 1:1000), which recognized the N-terminus of SNAP-25.
For the detection of the total GFP-tagged SNAP-25s, the
polyclonal antibody 116b was used (diluted 1:1000), which
recognized the C-terminus of SNAP-25. Phosphorylated SNAP-
25s and phosphorylated GFP–SNAP-25s were detected using
SN25Pi (diluted 1:600) [21]. The secondary antibody was an
anti-mouse or an anti-rabbit horseradish peroxidase-conjugated
antibody and visualization was obtained with the enhanced
chemiluminescence (ECL®) detection system (Amersham Bio-
sciences).

Isolation of rat islets of Langerhans

Rat islets of Langerhans were obtained as described previ-
ously [28]. Briefly, pancreata from eight rats were digested with
collagenase in Ca²⁺-containing Hank’s buffer and islets of
Langerhans were purified from exocrine tissue by discontinuous
density-gradient centrifugation (Histopaque 1077 from Sigma).
To allow for recovery from the isolation procedure, islets were
incubated overnight in Dulbecco's modified Eagle's medium
with 10% FCS, supplemented with 0.11 mg/ml penicillin,
110 units/ml streptomycin, 0.11 mg/ml sodium pyruvate and
8.3 mM glucose.

Islet secretion assay

At 1 day after isolation, islets were washed twice with Krebs–
Ringer bicarbonate buffer (KRB-Hepes, pH 7.4: 134 mM NaCl,
4.8 mM KCl, 1 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 5 mM NaHCO₃, 0.1 % BSA, 10 mM Hepes) containing 2.8 mM glucose. All islets were pre-incubated together for 1 h and then separated in batches of equal number (200–300 islets/condition) for further treatment with the different secretagogues. Islets were incubated in suspension in KRb-Hepes for 1 h in four different conditions: 2.8 mM glucose (basal), 16.7 mM glucose, 1 mM carbachol (CCh) or 1 μM PMA. In addition, islets were incubated with 16.7 mM glucose and 0.5 mM isobutylmethylxanthine (IBMX) and 10 μM forskolin. Basal and stimulated secretion media were collected after centrifugation of the islets at 70 g for 2 min and then media were again centrifuged to avoid any contamination with floating cells. Islets were directly lysed in Laemmli loading buffer without 2-mercaptoethanol, boiled and subjected to SDS/PAGE for protein detection. Insulin secretion was measured by RIA using rat insulin as the standard.

Secretion assay from permeabilized HIT cells

The secretion assay was performed as described previously [24]. The amount of C-peptide released from transfected cells was determined using a human C-peptide ELISA Kit (Dako, Cambridge, U.K.). For the determination of the cellular C-peptide content, HIT cells were lysed after the secretion assay in 0.2 ml/well solubilization buffer (20 mM Tris, pH 7.4; 1 mM EDTA; 1 mM EGTA; 150 mM NaCl; 0.5 % Triton X-100) and the C-peptide was determined after centrifugation in the cleared lysate using the same kit. This ELISA specifically recognizes the exogenous human C-peptide and does not cross-react with the endogenous hamster C-peptide of the HIT cells.

PKC inhibitors

After overnight culture at 37 °C, to allow for recovery from the isolation procedure, islets were pre-incubated for 1 h with either Gö 6976 (1 μM) or BIS (1 μM) in KRb-Hepes under basal conditions (2.8 mM). This incubation was then followed by a second 1 h incubation where the inhibitors were again added to the stimulated condition (16.7 mM glucose) or to the basal condition (2.8 mM glucose). Media were collected for insulin measurement and islets were lysed in Laemmli loading buffer to analyse the phosphorylation state of SNAP-25 by SDS/PAGE and Western blotting.

Immunoprecipitation of phosphorylated SNAP-25 in INS-1 cell lysates

INS-1 cells (at 0.5×10⁶ cells/dish) were lysed in 150 μl of modified RIPA buffer (10 mM Tris, pH 7.9, 150 mM NaCl, 0.5 % Triton X-100, 0.1 % SDS, 1 mM EDTA) containing 0.01 % phosphatase inhibitor cocktail 1 (Sigma-Fluka). The lysates were cleared by centrifugation at 100000 g for 45 min. The cleared lysates were then incubated at 4 °C overnight, with or without (as a control) 5 μl of antibody SN25Pi. The next day, Protein A-coupled Sepharose beads (50 μl of a 50 mg/ml slurry) were added to the lysates and incubated further for 3–5 h at room temperature. Beads were then spun down at 2700 g for 5 min, and supernatants were incubated for 2 h at room temperature with a further 5 μl of the SN25Pi antibody followed by 3–5 h incubation with fresh Sepharose beads. This latter procedure was repeated twice, in order to immunodeplete the lysate of phosphorylated SNAP-25. Lysates (input) and immunodepleted lysates (supernatant), as well as the pooled beads with the immunocomplexes (immune precipitate, I.P.), were boiled in Laemmli loading buffer. Only one-third from the total input and total supernatant were loaded and processed on SDS/PAGE under non-reducing conditions. Signals were quantified by densitometry and background values were subtracted from values of signals. The proportion of SNAP-25 that was phosphorylated was evaluated by quantifying the signals obtained with the total SNAP-25 antibody, taking the amounts loaded on the gels into consideration.

Presentation of data

Results are presented as means ± S.D. The level of significance for differences between groups was assessed using Student’s two-tailed t test for unpaired groups.

RESULTS

PMA induces phosphorylation of SNAP-25 at Ser¹⁸⁷ in INS-1 cells

In order to determine whether SNAP-25 can be phosphorylated by PKC at Ser¹⁸⁷ in the insulin secreting rat cell line INS-1, these cells were incubated in culture medium containing 1 μM PMA for 1 h and cell lysates were analysed by Western blotting under reducing conditions. Two different anti-SNAP-25 antibodies were used: a monoclonal antibody, directed against total SNAP-25 (SMI 81) and a polyclonal antibody, shown to be specific for SNAP-25 phosphorylated at Ser¹⁸⁷ (SN25Pi). Figure 1 shows that PMA treatment stimulated phosphorylation of SNAP-25 (increased signal with SN25Pi) without any change in the total cell content of SNAP-25 (unchanged signal with SMI81). Figure 2 shows a double-immunofluorescence study for total and phosphorylated SNAP-25 on PMA-treated INS-1 cells. INS-1 cells that were treated with PMA showed the presence of phosphorylated SNAP-25 at the plasma membrane (Figure 2D). Both PMA-treated and untreated cells showed a typical staining for total SNAP-25, primarily at the plasma membrane (Figures 2A and 2C).

Kinetics of phosphorylation and dephosphorylation of SNAP-25 in INS-1 cells

To examine the time-course of SNAP-25 (Ser¹⁸⁷) phosphorylation by PKC, INS-1 cells were incubated with 1 μM PMA for increasing periods of time, from 5 to 120 min. Phosphorylation of Ser¹⁸⁷ was already detectable at 5 min of incubation and the signal reached maximal intensity by 60 min (Figure 3). There was...
INS-1 cells were incubated in culture medium with or without 1 μM PMA for 1 h. Following PMA treatment, cells were fixed and permeabilized with ice-cold methanol and used for immunofluorescence staining. The cells were double-stained for total SNAP-25 with the antibody SMI81 (A and C) and for phosphorylated SNAP-25 with the antibody SN25Pi (B and D) and observed with a fluorescence microscope. Following stimulation with PMA, phosphorylated SNAP-25 is seen localized to the plasma membrane. Scale bar, 10 μm; same magnification for all images.

INS-1 cells were incubated in culture medium supplemented with 1 μM PMA for increasing periods of time, from 5 to 120 min. The control condition (C) is without PMA. After SDS/PAGE under reducing conditions and protein transfer, membranes were probed with the SN25Pi antibody, recognizing phosphorylated SNAP-25 (upper panel), or the SMI81 antibody, recognizing total SNAP-25 (lower panel). The phosphorylated SNAP-25 is visible after 5 min incubation with PMA and a maximal signal is reached at 1 h. The results show one representative experiment from a total of three independent experiments.

INS-1 cells were incubated with 100 nM PDA or 1 μM PMA for 40 min and then extensively washed with culture medium. After further incubation with culture medium for 0, 30 or 60 min, cells were lysed directly in loading buffer and subjected to SDS/PAGE under reducing conditions followed by Western blotting with the antibody against phosphorylated SNAP-25 (SN25Pi, upper panel) or against total SNAP-25 (SMI81, lower panel). Following stimulation with the water-soluble PDA, phosphorylation was rapidly reversible. This was not the case using the hydrophobic PMA. The results show one experiment from a total of two independent experiments.

INS-1 cells were incubated with 100 nM PDA or 1 μM PMA for 40 min and then extensively washed with culture medium. After further incubation with culture medium for 0, 30 or 60 min, cells were lysed directly in loading buffer and subjected to SDS/PAGE under reducing conditions followed by Western blotting with the antibody against phosphorylated SNAP-25 (SN25Pi, upper panel) or against total SNAP-25 (SMI81, lower panel). Following stimulation with the water-soluble PDA, phosphorylation was rapidly reversible. This was not the case using the hydrophobic PMA. The results show one experiment from a total of two independent experiments.

In cells treated with PMA, SNAP-25 phosphorylation at Ser187 is rapidly reversible.

In contrast, in cells treated with PDA, phosphorylation of SNAP-25 disappeared almost completely within 30 min of removal of the PKC activator. Given that there was no change in the total amount of SNAP-25, it was concluded that SNAP-25 phosphorylation at Ser187 is rapidly reversible.
forms of SNAP-25, regardless of the phosphorylation state of Ser187). In order to allow for direct blotting. The same membrane was probed first using the SN25Pi antibody (recognizing only phosphorylated SNAP-25) followed, after stripping, by the SMI81 antibody (recognizing all products (total I.P.) were analysed by SDS/PAGE under non-reducing conditions and Western blotting. The same membrane was probed first using the SN25Pi antibody (recognizing only phosphorylated SNAP-25) followed, after stripping, by the SMI81 antibody (recognizing all forms of SNAP-25, regardless of the phosphorylation state of Ser187). In order to allow for direct comparison, only one-third of lysates (input), one-third of immuno-depleted lysates (supernatant), but all total immunoprecipitated products (total I.P.) corresponding to pooled material from three rounds of immunoprecipitation, were loaded (see the Experimental section). Two exposure times are shown as indicated.

Proportion of SNAP-25 phosphorylated after PMA stimulation in INS-1 cells

The ratio of phosphorylated to non-phosphorylated SNAP-25 was evaluated by quantitative immunoprecipitation of the former. The relative amounts of phosphorylated and non-phosphorylated SNAP-25 were estimated by Western blotting. To this end, lysates of INS-1 cells treated with PMA were immunoprecipitated using SN25Pi, which is specific to the phosphorylated form of SNAP-25. Figure 5 shows that, using the specific anti-phospho-SNAP-25 antibody for the Western blot, there was little remaining phosphorylated SNAP-25 in the supernatant (Figure 5, upper panel), indicating that most had indeed been immunoprecipitated by this same antibody. As expected, the phosphorylated protein was recovered in the immune precipitate (total I.P.). The use of the other antibody for Western blotting allowed for estimation of the percentage of SNAP-25 that had been phosphorylated and immunoprecipitated (Figure 5, lower panel). Under these conditions, there was a residual signal in the supernatant. Measurement of the intensity of the respective signals (supernatant and I.P.) allowed us to estimate the percentage of total SNAP-25 that had been phosphorylated in response to PMA (note that this estimate takes into consideration the relative amounts of material loaded on gels for Western blots; see the legend to Figure 5). Although this value never exceeded 35%, for unknown reasons, it varied greatly from one experiment to the next (12.6, 23.8 and 35.4 %, respectively for three independent experiments).

Soluble SNAP-25 is not phosphorylated at Ser187

Palmitoylation of SNAP-25 cysteine residues is implicated in its association with the plasma membrane. We have shown previously that mutation of two or more of these four cysteine residues results in almost complete displacement of SNAP-25 from membranes to the cytosol [24]. These mutants can thus be used to determine whether membrane association is necessary for SNAP-25 phosphorylation. To this end, INS-1 cells were transfected with four different SNAP-25 constructs: GFP–SNAP-25wt localized totally at the plasma membrane; GFP–SNAP-25 single cysteine mutant, (GFP–SNAP-25Cys<sup>80</sup> → Ala), which is 50% cytosolic; GFP–SNAP-25Cys<sup>85</sup> → Ala/Cys<sup>88</sup> → Ala and GF–SNAP-25Cys<sup>80</sup> → Ala/Cys<sup>88</sup> → Ala/Cys<sup>90</sup> → Ala/Cys<sup>92</sup> → Ala which are both cytosolic. The addition of GFP to the N-terminus of SNAP-25 is without effect on either subcellular localization or biological activity [24], but allows for separation from endogenous SNAP-25 by virtue of increased molecular mass. After transfection, the cells were treated with PMA and analysed by Western blotting (Figure 6). All mutants were equally expressed in INS-1 cells (Figure 6B). In the presence of PMA, only the GFP–SNAP-25wt and the single cysteine mutant, as well as the endogenous SNAP-25, were phosphorylated. Phosphorylation of the single cysteine mutant was, however, decreased relative to the wt fusion protein. Neither of the two cytosolic mutants were phosphorylated to any detectable extent; this is taken to indicate that association with the plasma membrane is needed for phosphorylation. Although the possibility that conformational changes due to the cysteine mutations could interfere with phosphorylation cannot be totally excluded, this is considered most unlikely on theoretical grounds.

In order to confirm the specificity of the SN25Pi antibody, INS-1 cells were transfected with GFP–SNAP-25Ser<sup>187</sup> → Ala or GFP–SNAP-25Ser<sup>187</sup> → Asp and again stimulated with PMA. As shown in Figure 6, neither of these mutants, which cannot be phosphorylated at Ser<sup>187</sup>, are recognized by the antibody SN25Pi.

SNAP-25 isoforms a and b are phosphorylated to a different extent

We have shown previously that insulin-secreting cells express both isoforms of SNAP-25, SNAP-25a and SNAP-25b, and that both are able to function in insulin secretion [23]. In order to investigate whether both isoforms are phosphorylated, INS-1 cells were transfected with N-terminal-tagged SNAP-25a or SNAP-25b constructs. With comparable amounts of the two isoforms that were expressed, we observed a significant difference of phosphorylation at Ser<sup>187</sup> between both isoforms, following 1 h stimulation with PMA (Figure 7A). Quantification of four independent experiments revealed that there was a 1.8-fold greater phosphorylation of SNAP-25b compared with SNAP-25a (Figure 7B).

Glucose and CCh induce SNAP-25 phosphorylation in primary rat islets

The results presented above show clearly that SNAP-25 phosphorylation is inducible in INS-1 cells by PMA. It was felt important to test a more physiological stimulus using primary β-cells rather than a transformed cell line. To this end, islets were isolated from rat pancreas and incubated for 1 h under four different conditions: 2.8 mM glucose (basal), 16.7 mM glucose, 1 mM CCh or 1 μM PMA (positive control). Using the same analytical approach as for INS-1 cells, the results showed that high glucose levels, as well as CCh, can induce SNAP-25 phosphorylation at Ser<sup>187</sup> in islets when compared with the non-stimulated basal condition, albeit to a lesser extent than PMA. Stimulation of islets with high glucose levels plus IBMX and forskolin, which indirectly activates PKA by raising cAMP levels, does not increase further SNAP-25 phosphorylation

SNAP-25 phosphorylation in insulin-secreting cells

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Figure 6  Soluble SNAP-25 is not phosphorylated after stimulation with PMA

INS-1 cells were transfected with an empty vector, GFP–SNAP-25 wt or one of the GFP–SNAP-25 cysteine mutants. Two days after transfection, cells were incubated in 1 μM PMA for 1 h. Cells were lysed in loading buffer and equal amounts were used for two Western blots: A, phosphorylated SNAP-25 was detected with the antibody SN25PI; B, total SNAP-25 with the polyclonal 116b antibody. The positions of GFP–SNAP-25 wt and cysteine mutants as well as that of the endogenous SNAP-25 are indicated by arrows. Phosphorylation of the single cysteine mutant is decreased and that of the double and quadruple mutants is abolished. Neither of the two molecules mutated at Ser187 (GFP–SNAP-25Ser187→Ala and GFP–SNAP-25Ser187→Asp) was recognized by the SN25PI antibody, in confirmation of the specificity of this antibody for phosphorylation of this residue. The results show one representative experiment from a total of three independent experiments.

compared with glucose alone (Figure 8). Quantification by densitometry indicated that both high glucose levels and CCh stimulated phosphorylation approx. twice as much as basal levels, whereas PMA elicited a 5-fold stimulation (Figure 9).

The extent of SNAP-25 phosphorylation does not correlate with that of insulin secretion

If SNAP-25 phosphorylation plays an important physiological role in regulated insulin secretion, a correlation would be expected between the extent of insulin secretion and that of SNAP-25 phosphorylation. Therefore we measured the phosphorylation state of SNAP-25 and the amount of insulin secreted from the same isolated rat islets stimulated with glucose (16.7 mM), CCh (1 mM) or PMA (1 μM) for 1 h. In such a static secretion assay, insulin release from islets that were treated with high glucose levels was 2.5-fold greater than that from islets treated with CCh (Figure 9). SNAP-25 phosphorylation, however, was no different under these two conditions. Furthermore, islets treated with PMA show 5–6-fold more phosphorylated SNAP-25 but there was no statistically significant difference in insulin secreted with PMA compared with 16.7 mM glucose (Figure 9).

High glucose levels induce SNAP-25 phosphorylation through the activation of conventional and non-conventional PKC isoforms

Conventional (Ca2+-dependent) and novel (Ca2+-independent), as well as atypical [Ca2+-independent forms that do not bind diacylglycerol (DAG)], PKC isoforms are expressed in pancreatic cells [6]. In order to investigate which isoforms are involved in SNAP-25 phosphorylation, isolated islets were treated with two different PKC inhibitors, Gö 6976 preferentially inhibits the conventional PKC isoforms, but not the novel or the atypical ones; however, BIS (at 1 μM) is known to be a universal PKC inhibitor. Islets were incubated with or without 1 μM Gö 6976 or 1 μM BIS throughout both the basal (2.8 mM glucose) and stimulated (16.7 mM glucose) periods. The results in Figure 10(A) show that, in the basal condition, both PKC inhibitors were equally effective in lowering SNAP-25 phosphorylation. The remaining phosphorylation of SNAP-25 after inhibition by BIS under basal and glucose-stimulated conditions was similar, whereas that, after inhibition by Gö, was higher under glucose-stimulated conditions. However, phosphorylation at high glucose levels was not significantly different in the presence of either inhibitor. This suggests that phosphorylation of SNAP-25 in rat islets under basal and stimulated conditions occurs through the activation of conventional and non-conventional PKC isoforms. Furthermore, the quantification of insulin release from treated or untreated islets (Figure 10B) showed that the PKC inhibitors BIS or Gö 6976 do not inhibit glucose-induced insulin secretion, thereby again suggesting that phosphorylated SNAP-25 is not necessary for efficient insulin secretion.

Investigation of the functional activity of the toxin-resistant SNAP-25bTRSer187 mutants in Ca2+-stimulated exocytosis from botulinum neurotoxin E (BoNT/E)-treated HIT cells

In order to analyse directly the possible importance of Ser187 in the process of exocytosis, the ability of TR GFP–SNAP-25 phosphorylation, isolated islets were treated with two different PKC inhibitors, Gö 6976 preferentially inhibits the conventional PKC isoforms, but not the novel or the atypical ones; however, BIS (at 1 μM) is known to be a universal PKC inhibitor. Islets were incubated with or without 1 μM Gö 6976 or 1 μM BIS throughout both the basal (2.8 mM glucose) and stimulated (16.7 mM glucose) periods. The results in Figure 10(A) show that, in the basal condition, both PKC inhibitors were equally effective in lowering SNAP-25 phosphorylation. The remaining phosphorylation of SNAP-25 after inhibition by BIS under basal and glucose-stimulated conditions was similar, whereas that, after inhibition by Gö, was higher under glucose-stimulated conditions. However, phosphorylation at high glucose levels was not significantly different in the presence of either inhibitor. This suggests that phosphorylation of SNAP-25 in rat islets under basal and stimulated conditions occurs through the activation of conventional and non-conventional PKC isoforms. Furthermore, the quantification of insulin release from treated or untreated islets (Figure 10B) showed that the PKC inhibitors BIS or Gö 6976 do not inhibit glucose-induced insulin secretion, thereby again suggesting that phosphorylated SNAP-25 is not necessary for efficient insulin secretion.

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SNAP-25 phosphorylation in insulin-secreting cells

**Figure 7** SNAP-25a and SNAP-25b are both phosphorylated, but to a different extent

INS-1 cells were transfected with either myc–SNAP-25a or myc–SNAP-25b constructs. Two days after transfection, cells were incubated with or without 1 μM PMA for 1 h and lysed directly in loading buffer. Total lysates were used for SDS/PAGE under reducing conditions and Western blotting. **A**, Phosphorylated SNAP-25 was detected by SN25Pi (upper panel) and total SNAP-25 was detected by SMI81 (lower panel). The positions of myc–SNAP-25 and endogenous SNAP-25 are indicated by arrows. **B**, Phosphorylation of each isoform was quantified after normalization of the phosphorylated form with the total amount of myc–SNAP-25. Results are from four independent experiments.

**Figure 8** Glucose and CCh induce SNAP-25 phosphorylation in islets

Equal numbers of islets were incubated under basal conditions (2.8 mM glucose) for 1 h, followed by a second 1 h incubation with basal glucose (2.8 mM), high glucose (16.7 mM), CCh (1 mM) and PMA (1 μM). Media from basal and stimulated conditions were collected and the amount of insulin was quantified by RIA. The results of the insulin measurements are expressed as fold stimulation relative to basal release. The intensities of the immunoreactive bands of phosphorylated SNAP-25 (Figure 8) were quantified by densitometry and normalized with the values obtained for total SNAP-25. Results are for stimulation of phosphorylation relative to basal glucose from three independent experiments. The amount of insulin released under basal conditions was 0.44 ± 0.18 ng/h per islet.

25bTRSer<sup>187</sup> → Ala and GFP–SNAP-25bTRSer<sup>187</sup> → Asp mutants to reconstitute insulin secretion was assessed in cells in which endogenous SNAP-25 was inactivated by BoNT/E [24,29]. Since both isoforms are able to reconstitute insulin secretion to the same extent [23], but the SNAP-25b isoform is more phosphorylated after PMA treatment, the SNAP-25b isoform was used for these experiments. The GFP–SNAP-25bTRwt has been shown previously to be fully functional compared with non-tagged SNAP-25bTR [24]. Transformed HIT cells had to be used for this technically demanding approach, since these cells can be readily permeabilized while remaining attached to the culture dish and the ELISA, because human C-peptide does not cross-react with hamster C-peptide. HIT cells were co-transfected with cDNA for GFP–SNAP-25bTRwt or a GFP–SNAP-25bTRSer<sup>187</sup> mutant and human pro-insulin. Human pro-insulin is used as a marker for secretion from the subpopulation of transfected cells. The quantity of C-peptide (the by-product of conversion of human pro-insulin to insulin) secreted from the transfected cells was determined by ELISA using an antibody that specifically recognizes human, but not hamster, C-peptide. After permeabilization of HIT cells with streptolysin-O, cells were incubated with or without BoNT/E before insulin secretion was stimulated with high-Ca<sup>2+</sup> buffer. Under these conditions, GFP–SNAP-25bTRwt replaces the endogenous, cleaved SNAP-25 and reconstitutes insulin secretion almost completely (Figure 11). The GFP–SNAP-25bTRSer<sup>187</sup>Ala and the GFP–SNAP-25bTRSer<sup>187</sup>Asp mutants are both able to reconstitute insulin secretion as effectively as the wt.

**DISCUSSION**

Analysis of the amino acid sequence of SNAP-25 reveals a putative PKC consensus site in the C-terminal region at Ser<sup>187</sup>. This residue was identified as being phosphorylated in PC12 cells in response to stimulation with PMA or after NGF treatment [20,21,30]. We considered this phosphorylation site to be of particular interest because it is localized in between the sites of

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Figure 10 High glucose levels induce SNAP-25 phosphorylation through the activation of conventional and non-conventional PKC isoforms

Islets were pre-incubated for 1 h under basal glucose conditions (2.8 mM) containing the PKC inhibitors BIS (1 μM) or Gö6976 (1 μM). This was followed by a second 1 h incubation under either basal (2.8 mM glucose) or stimulated (16.7 mM glucose) conditions in the continued presence of the inhibitors. Media from basal and stimulated conditions were collected and the amount of insulin was quantified by RIA. Islets were lysed in loading buffer and used for SDS/PAGE under non-reducing conditions and Western blotting. A, Membranes were first incubated with the antibody against phosphorylated SNAP-25 (SN25Pi) and afterwards with the antibody recognizing total SNAP-25 (SMI81). The intensities of the immunoreactive bands of phosphorylated SNAP-25 were quantified by densitometry and normalized to values obtained for total SNAP-25. The results of the quantification are illustrated as fold stimulation relative to the value obtained in the basal glucose condition, which was set as 1. Results are from three independent experiments.

B, Insulin secretion measured from the same islets. Results are from three independent experiments, except for stimulated conditions with Gö6976, where there were two independent experiments. N.S., not significant.

Figure 11 Reconstitution of Ca\(^2+\)-induced insulin secretion by GFP–SNAP-25bTRwt compared with GFP–SNAP-25bTRSer\(^{187}\) mutants

HIT cells were transiently co-transfected with human pro-insulin and the empty vector, GFP–SNAP-25bTRwt or one of the GFP–SNAP-25bTRSer\(^{187}\) mutants. Two days after transfection, cells were permeabilized with streptolysin-O and incubated without (−) or with (+) 30 nM BoNT/E for 8 min. The medium was then replaced by high (+/+) or low (−/−) Ca\(^2+\)-containing buffers for a secretion period of 7 min. Insulin release from transfected cells was estimated by measuring the amount of human C-peptide released into the medium. Results are from three observations from two experiments for the Ser\(^{187}\)→Ala mutant and one for the Ser\(^{187}\)→Asp mutant. A, Reconstitution by the GFP–SNAP-25bTRSer\(^{187}\)→Ala mutant. B, Reconstitution by the GFP–SNAP-25bTRSer\(^{187}\)→Asp mutant.

Cleavage of the SNAP-25 inactivating botulinum toxin proteases BoNT/E (Arg\(^{188}\)_Ile\(^{181}\)) and BoNT/A (Gln\(^{197}\)–Arg\(^{198}\)) [31]. Recent evidence has shown further that this domain mediates a Ca\(^{2+}\)-dependent interaction between synaptotagmin and the SNARE protein complex [32]. Therefore phosphorylation at this site could directly influence such protein–protein interactions and thereby regulate the exocytotic event. To date, only one study has reported SNAP-25 phosphorylation in insulin-secreting cells. This was, however, at an unidentified tyrosine residue, rather than a serine residue, and in response to a combination of glucose and glucagon-like peptide-1 (GLP-1) stimulation. Furthermore, neither identification of the kinase responsible for this phosphorylation nor demonstration of direct involvement of this phosphorylation event in insulin secretion was achieved [33].

Using an antibody specifically recognizing the phosphorylation of SNAP-25 at Ser\(^{187}\), we have demonstrated that this particular
phosphorylation event does occur in insulin-secreting cells. Both isoforms of SNAP-25 are phosphorylated in the presence of PMA, with the latter being phosphorylated 1.8-fold more. The physiological significance of this difference remains unclear. The marked and rapid phosphorylation of membrane-anchored SNAP-25 at Ser187 in INS-1 cells in response to PMA clearly implicates PKC in the phosphorylation event at Ser187. This is in agreement with the previously observed in vitro phosphorylation of SNAP-25 by PKC [20]. The mere observation of phosphorylation in response to pharmacological stimulation of transformed cells does not of itself indicate the physiological relevance of the event or its possible role in insulin secretion. We therefore investigated further to see if this phosphorylation is inducible by more physiological stimulation of primary pancreatic islet cells and if it is correlated with insulin secretion from such cells. SNAP-25 phosphorylation in primary rat islets was stimulated by glucose as well as by CCh. However, glucose, together with IBMX and forskolin, does not increase further the phosphorylation of SNAP-25, but does increase insulin secretion from islets (results not shown). Therefore, activation of PKA does not seem to have an important role in the phosphorylation of Ser187. The activation of islet PKC by glucose has been amply documented in the literature. The nutrient secretagogue glucose activates PKC through de novo formation of phosphatidic acid and DAG [34]. In contrast, receptor-operated non-nutrient secretagogues, such as CCh, activate PKC via the phospholipase C pathway leading to hydrolysis of phosphatidylinositol bisphosphate and generation of DAG [35,36]. The induction of SNAP-25 phosphorylation through glucose and CCh could indicate that this phosphorylation is because of activation of the same set of DAG-dependent PKC isoforms. Furthermore, activation of PKC by PMA [37] or glucose [38–40] is accompanied by redistribution of the PKC α and ε isoforms from the cytosol to the plasma membrane. This could indicate further that phosphorylation at Ser187 is specifically due to the activation of these PKC isoforms, since only membrane-associated SNAP-25 is phosphorylated. Using the PKC inhibitors Go 6976 and BIS, we have shown that both are able to inhibit basal and glucose-stimulated SNAP-25 phosphorylation. This is taken to indicate that glucose stimulation of SNAP-25 phosphorylation at Ser187 involves both conventional and non-conventional isoforms.

It has been shown that one physiological consequence of SNAP-25 phosphorylation is a decrease in the interaction with its binding partner syntaxin 1A, leading to the hypothesis that this could influence exocytosis [20]. In our model system, using a static secretion assay, we observed that the extent of insulin secretion does not correlate with that of SNAP-25 phosphorylation and, more convincingly, our secretion/reconstitution assay shows that the TR Ser187 mutants are able to reconstitute insulin secretion efficiently in HIT cells, where endogenous SNAP-25 has been inactivated by BoNT/E. These results are in agreement with a recent study, which showed that, in PC12 cells, phosphorylation has only a minor importance in PMA-potentiated high K+ level-induced neurotransmitter release (inhibition of PKC by staurosporine suppressed SNAP-25 phosphorylation completely but decreased neurotransmitter release only to a small extent) [30]. It remains possible that phosphorylation may be important for a very minor and early phase of secretion, perhaps corresponding to release from the so-called ‘immediately releasable’ pool of granules [41] and consequent to the establishment of micro-domains with an elevated capacity for rapid granule docking/fusion. Unfortunately, addressing this possibility directly would depend upon real-time analysis of SNAP-25 phosphorylation. This is not, at present, feasible. Another possibility is that SNAP-25 phosphorylation may modulate some other exocytotic event. This could include exocytosis of vesicles other than large dense-core insulin-containing granules, as has been proposed for phosphorylated syntaxin in the rat brain [42].

In conclusion, SNAP-25 is phosphorylated at Ser187 in insulin-secreting cells in response to stimuli including glucose. The amount of SNAP-25 that is phosphorylated after PMA stimulation does not exceed 35%, and very much less is phosphorylated following stimulation by glucose. The toxin-resistant GFP–SNAP-25bTRSer187→Ala and GFP–SNAP-25bTRSer187→Asp mutants are both able to reconstitute insulin secretion. This, and the lack of correlation between SNAP-25 phosphorylation and insulin secretion from islets, suggest that phosphorylation at Ser187 is not implicated in the regulation of insulin secretion. Further studies are needed to determine whether the phosphorylation state of SNAP-25 is important for other aspects of β-cell function.

We thank Dr Domenico Bosco for many helpful discussions. This work was supported by the Swiss National Science Foundation grant No. 3200-061776.00.

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232 C. Gonelle-Gispert and others


Received 11 June 2002/29 July 2002; accepted 6 August 2002
Published as BJ Immediate Publication 6 August 2002, DOI 10.1042/BJ20020896

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