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Differential phosphorylation of SNAP-25 in vivo by protein kinase C and protein kinase A

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Abstract SNAP-25 is a key protein required for the fusion of synaptic vesicles with the plasma membrane during exocytosis. This study establishes that SNAP-25 is differentially phosphorylated by protein kinase C and protein kinase A in neuroendocrine PC12 cells. Using phosphopeptide mapping and site-directed mutagenesis we identified both Thr138 and Ser187 as the targets of SNAP-25 phosphorylation by protein kinase C and Thr138 as the exclusive site of SNAP-25 phosphorylation by protein kinase A in vivo. Finally, despite published data to the contrary, we demonstrate that stimulation of regulated exocytosis under physiological conditions is independent of a measurable increase in SNAP-25 phosphorylation in PC12 cells.

Key words: SNAP receptor; Synaptosome-associated protein of 25 kDa; Exocytosis; Phosphorylation

1. Introduction

Neurotransmitters and hormones are released from neuronal cells by calcium-triggered exocytosis of secretory vesicles. Extensive research on the molecular mechanisms underlying exocytosis demonstrated that proteins termed SNAREs (soluble NSF attachment protein (SNAP) receptors) are essential mediators of synaptic vesicle fusion with the presynaptic plasma membrane [1,2]. The membrane-associated SNARE complex consists of interactions between SNAREs present on vesicles (synaptobrevin family) and on target membranes (syntaxin and synaptosome-associated protein of 25 kDa (SNAP-25) families [3]).

Protein phosphorylation represents an important mechanism for regulating protein–protein interactions and numerous studies have demonstrated its role in the control of neurotransmission [4]. While there have been studies showing that the t-SNARE SNAP-25 can be phosphorylated [5–9], the physiological relevance of SNARE phosphorylation remains unclear. Although extreme conditions of neuronal stimulation have been associated with SNAP-25 phosphorylation [8,9], there are no data examining the importance of SNAP-25 phosphorylation under physiologically relevant conditions of neurotransmitter release. In the present study we have biochemically identified the sites of SNAP-25 phosphorylation in neuroendocrine PC12 cells stimulated with phorbol 12-myristate 13-acetate (PMA) or forskolin and have examined the extent to which SNAP-25 phosphorylation correlates with neurotransmitter release in vivo.

2. Materials and methods

2.1. Cell culture and transfections

PC12 cells (clone PC12G2, from Dr. Steven Sabol, National Heart, Lung and Blood Institute, NIH, Bethesda, MD, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (HyClone Laboratories, Logan, UT, USA), 5% horse serum (Sigma, St. Louis, MO, USA), 20 mM HEPES (pH 7.5) and 50 ng/ml gentamicin. Cells were maintained as subconfluent monolayers and passaged with 150 mM NaCl, 0.5 mM EDTA, 20 mM HEPES (pH 7). HeLa cells were cultured and transiently transfected using Lipofectamine (Gibco, Grand Island, NY, USA) as described previously [10].

2.2. Site-directed mutagenesis

SNAP-25 Ala mutants of Thr138, Ser187, and a Thr138/Ser187 double mutant were generated using the Quickchange mutagenesis kit (Stratagene, La Jolla, CA, USA) with pcDNA3-SNAP-25b [11] as template according to the manufacturer's instructions. All mutations were confirmed by automated sequence analysis using an ABI sequencer.

2.3. Metabolic labeling of cells

HeLa and PC12 cells were plated at 1.5×10^6 and 3×10^6 cells per 10 cm dish respectively. Cells were starved the next day for 15 min in phosphate-free DMEM containing 3% dialyzed calf serum (HyClone Laboratories), 20 mM HEPES (pH 7.5), and gentamicin. Cells were labeled with 0.5 mCi [^{32}P]orthophosphate (NEN Life Science, Boston, MA, USA) for 4 h in 1 ml phosphate-free DMEM. PC12 cells were then stimulated for secretion as described below. PC12 and transfected HeLa cells were also incubated with 1 μM ionomycin, 160 nM PMA or 40 μM forskolin for 30 min. Cells were washed with ice-cold Hanks' buffered saline solution then frozen until further use.

2.4. Immunoprecipitation and electrophoresis

Immunoprecipitation was carried out as described previously [10]. Briefly, cells were lysed for 1 h in ice-cold lysis buffer containing 1% Triton X-100 as well as protease and phosphatase inhibitors. Specific immunoprecipitation was performed for 2 h at 4°C using the anti-SNAP-25 monoclonal antibody (mAb) SMI81 (Sternberger Monoclonals, Lutherville, MD, USA). Proteins from the immunoprecipitates were separated on a 12.5% SDS–PAGE and radiolabeled molecules were visualized by autoradiography and the extent of phosphorylation was quantitated by phosphorimager analysis. For tryptic digestion or phosphoamino acid analysis, proteins were transferred to nitrocellulose (0.2 μm, Schleicher and Schuell, Keene, NH, USA) or polyvinyl-

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Abbreviations: PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; mAb, monoclonal antibody; NA, noradrenaline; SNAP, soluble NSF attachment protein; SNAP-25, synaptosome-associated protein of 25 kDa; SNARE, SNAP receptor

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idene difluoride (PVDF) (0.2 μm; Bio-Rad, Hercules, CA, USA) membranes which were exposed to a phosphorscreen overnight.

2.5. Phosphoamino acid analysis

Phosphoamino acid analysis was performed as described earlier [7]. SNAP-25 on the PVDF membrane was hydrolyzed in 6 N HCl for 2 h at 110°C. The hydrolysate was then vacuum dried and amino acids resuspended in water. An aliquot and phosphoamino acid standards were separated by thin layer chromatography (plates from E.M. Science, Gibbstown, NJ, USA, No. 5577) with isobutyric acid:0.5 M NH_4OH (5:3 v/v). Standards were visualized with 0.2% ninhydrin (Sigma) while [^{32}P]amino acids were analyzed by autoradiography.

2.6. Tryptic digestion

Nitrocellulose pieces containing SNAP-25 were incubated 1 h at 37°C in 1% PVP-40 (Sigma), washed twice with 0.4% NH_4HCO_3 (pH 8.0) and incubated overnight with 1 mg/ml trypsin at 37°C in the same buffer. The released peptides were dried in a speed-vac and washed with H_2O several times, then resuspended in 5–10 μl H_2O. Aliquots were analyzed by autoradiography after thin layer chromatography using butanol-1/pyridine/acetic acid/H_2O (5:3:1:4 v/v).

2.7. [^{3}H]Noradrenaline ([^{3}H]NA) secretion

Secretion experiments from [^{3}H]NA-loaded PC12 cells was performed using 59 mM KCl or 60 μM nicotine in Locke solution [12] or using 40 μM forskolin, 1 μM ionomycin, or 160 nM PMA in PC12 culture medium. Basal secretion was identical in each solution. [^{3}H]NA release was determined by measuring the radioactivity present in the medium and in cells after precipitation with 10% trichloroacetic acid. Release of [^{3}H]NA is expressed as a percentage of total radioactivity taken up by the cells.

2.8. Statistics

For quantitative experiments, statistical analyses were carried out using the unpaired Student's *t*-test. Results were considered significant when a *P* value of less then 0.05 was obtained.

3. Results

3.1. Activation of protein kinase C (PKC) and cAMP-dependent protein kinase (PKA) triggers in vivo phosphorylation of SNAP-25

To address the role of SNAP-25 phosphorylation in vivo, we set out to unambiguously identify the SNAP-25 phosphorylation sites using phosphopeptide analysis and site-directed mutagenesis. We metabolically labeled cells with [^{32}P]orthophosphate to examine the phosphorylation status of endogenous SNAP-25 in rat pheochromocytoma PC12 cells or SNAP-25-transfected HeLa cells (which normally do not express SNAP-25). In untreated (control) cells, basal phosphorylation of SNAP-25 could be detected in each cell type (Fig. 1A). Activation of PKC with PMA and PKA with forskolin increased the phosphorylation level of SNAP-25 in both cell types. Phosphopeptide mapping of gel-purified and trypsin-digested SNAP-25 showed that PMA treatment resulted in the generation of two major tryptic phosphopeptides (single and double asterisk), while forskolin induced the phosphorylation of only one fragment (single asterisk) in both PC12 and HeLa cells (Fig. 1B). These data demonstrate that the same tryptic fragments are phosphorylated in both cells types, thereby allowing us to use expression of SNAP-25 point mutants in HeLa cells to map the SNAP-25 phosphorylation sites.

3.2. Identification of SNAP-25 phosphorylation sites in vivo

Phosphoamino acid analysis of untreated and PMA-stimulated SNAP-25-transfected HeLa cells revealed that SNAP-25 is phosphorylated on both Ser and Thr residues (Fig. 2A). To

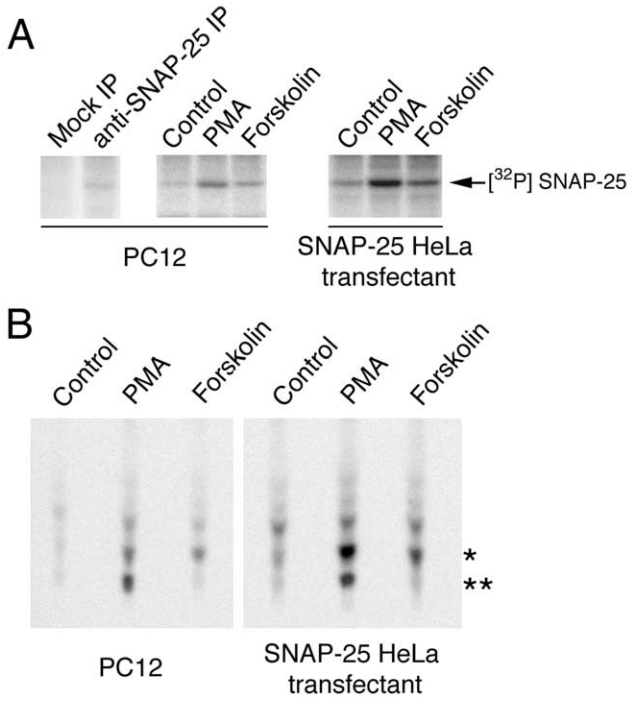

Fig. 1. SNAP-25 is phosphorylated in vivo. A: Basal and induced SNAP-25 phosphorylation. PC12 cells (left panel) and SNAP-25-expressing HeLa cells (right panel) were loaded with ^{32}P for 4 h prior to stimulation for 30 min in medium alone (Control) or medium containing either 160 nM PMA or 40 μM forskolin. SNAP-25 was isolated by immunoprecipitation and analyzed by SDS–PAGE and autoradiography. In the far left panel, immunoprecipitation from lysates derived from untreated control cells was performed using either isotype-matched irrelevant mAb (Mock IP) or SNAP-25 mAb highlighting the specificity of the SNAP-25 mAb for immunoprecipitation of [^{32}P]SNAP-25. B: [^{32}P]Phosphopeptide analysis. PC12 cells (left panel) and SNAP-25-transfected HeLa cells (right panel) were labeled with ^{32}P for 4 h prior to stimulation for 30 min in medium alone (Control) or medium containing either 160 nM PMA or 40 μM forskolin. SNAP-25 was isolated, subjected to trypsin digestion, and analyzed by thin layer chromatography as described in the text.

identify the PMA-induced as well as the forskolin-induced phosphorylation sites, we generated Ala mutants of SNAP-25 at positions Thr138 (an in vitro PKA site [7]), Ser187 (the only described PKC site [5]), and a double Ala mutant of both Ser187 and Thr138. These mutants were transfected into HeLa cells, subsequently labeled with ^{32}P, and stimulated with PMA (Fig. 2B) or forskolin (Fig. 2C). Immunoblot analysis confirmed that all mutants were expressed at similar levels (data not shown). As shown above, stimulation of PKC activity with PMA gave rise to two prominent tryptic phosphopeptides in wild-type SNAP-25. Replacement of Thr138 with Ala resulted in the selective elimination of the upper spot, demonstrating that this phosphopeptide contains phospho-Thr138 only (Fig. 2B). Moreover, forskolin failed to stimulate phosphorylation of the SNAP-25 Thr138Ala mutant in HeLa cells (Fig. 2C), further demonstrating that Thr138 is phosphorylated by PKA in vivo. Replacement of Ser187 with Ala resulted in the selective elimination of the lower spot, indicating that this peptide contains phospho-Ser187. Complete elimination of both PMA-induced tryptic phosphopeptides was only achieved in the Thr138/Ser187 double mutant, demonstrating that both of these residues are phosphorylated when cells are stimulated with PMA (Fig. 2B). Importantly, in

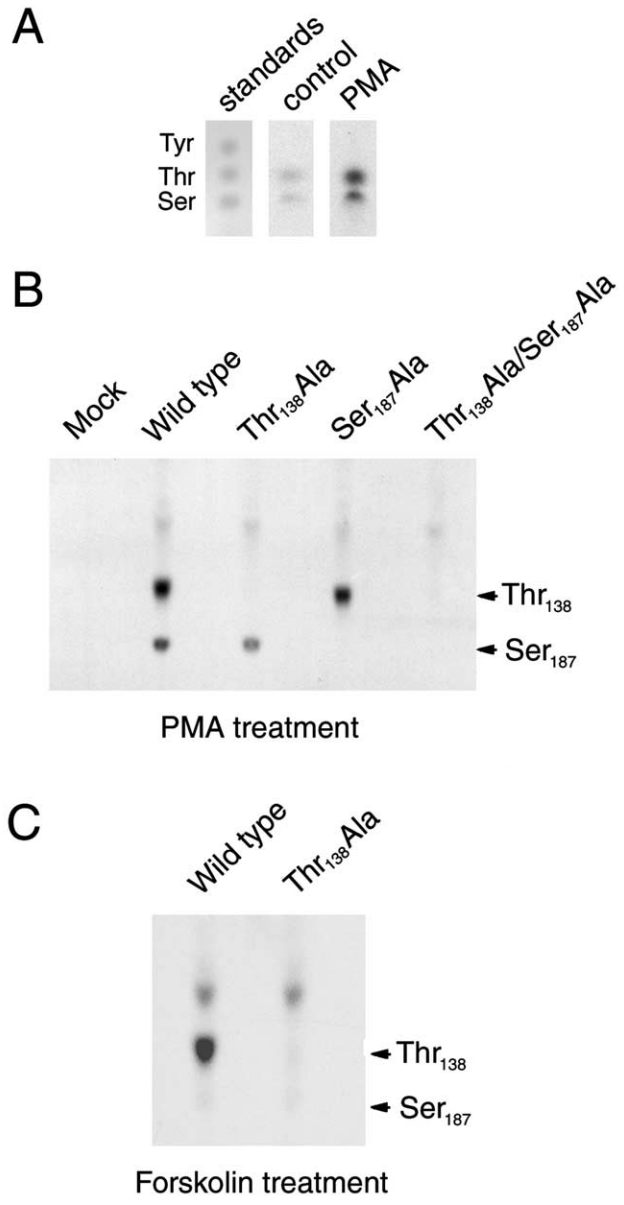

Fig. 2. Identification of SNAP-25 phosphorylation sites. A: Phosphoamino acid analysis. SNAP-25 from ^{32}P-labeled transfected HeLa cells treated with PMA was immunoprecipitated from cell lysates, isolated by SDS–PAGE, subjected to total acid hydrolysis, and phosphoamino acids were analyzed by thin layer chromatography. Radiolabeled amino acids were visualized by autoradiography and identified by comparison to phosphoamino acid standards stained with ninhydrin. B,C: [^{32}P]Phosphopeptide analysis of SNAP-25 phosphorylation mutants. HeLa cells were transfected with the pcDNA3 control vector (mock) or pcDNA3 containing wild-type, Thr138Ala, Ser187Ala, Thr138Ala/Ser187Ala forms of SNAP-25. After labeling with ^{32}P cells were stimulated for 30 min with PMA (B) or forskolin (C). SNAP-25 was isolated by immunoprecipitation and tryptic phosphopeptides were analyzed as described in Fig. 1.

all mutants examined background labeling remained unaltered (Fig. 2B,C), revealing further that Thr138 and Ser187 do not contribute to basal SNAP-25 phosphorylation.

3.3. Effects of regulated secretion on SNAP-25 phosphorylation

Since SNAP-25's major physiological function is related to neurotransmitter secretion, we set out to determine if this protein was inducibly phosphorylated by various secretagogues in PC12 cells. Allowing calcium influx using ionomycin, inducing membrane depolarization by KCl, and stimulating nicotinic acetylcholine receptors with nicotine all induced robust neurotransmitter release, while forskolin or PMA treatment alone failed to induce any secretion. In contrast, co-treatment with PMA nearly doubled the amount of [^{3}H]NA released from ionomycin-stimulated cells (Fig. 3A).

In a parallel series of experiments, the phosphorylation status of SNAP-25 was examined in ^{32}P-loaded PC12 cells that were stimulated for exocytosis. Treatment with forskolin in-

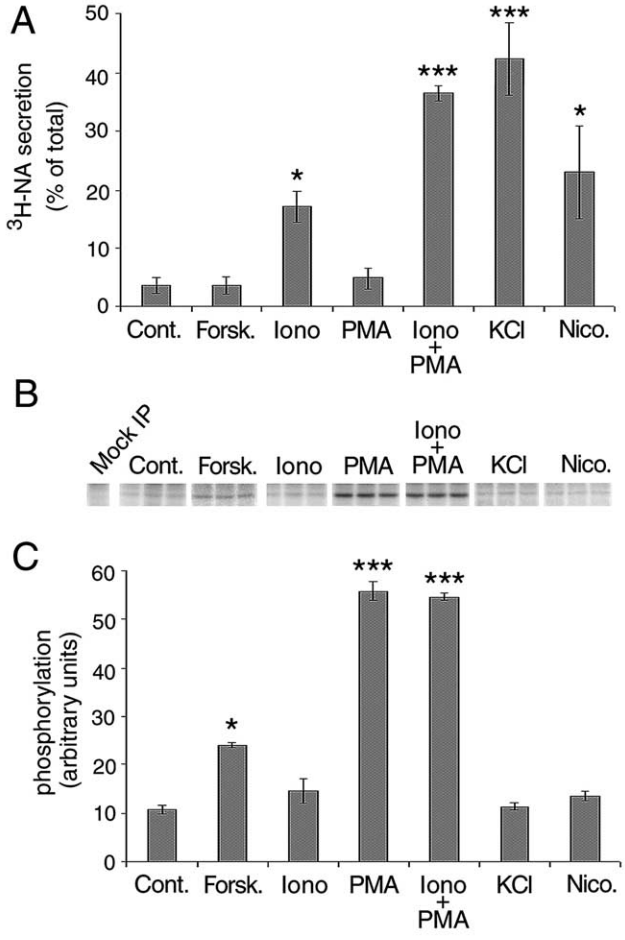

Fig. 3. Relationship between regulated secretion and SNAP-25 phosphorylation in PC12 cells. A: Secretion of PC12 cells in response to various secretagogues. PC12 cells were loaded with [^{3}H]NA for 2 h before stimulation for 20 min with buffer alone (Control), forskolin, ionomycin, PMA, ionomycin and PMA together, KCl, or nicotine. Secretion was calculated as a percentage of [^{3}H]NA released to the total amount of cell-associated [^{3}H]NA. $*P<0.05$, $***P<0.001$ as compared to the control sample. B: SNAP-25 phosphorylation in response to various secretagogues. PC12 cells were loaded with ^{32}P for 4 h and stimulated for 20 min with each of the secretagogues listed above. Each treatment was performed in triplicate. The cells were lysed and immunoprecipitations with isotype-matched irrelevant mAb (Mock IP, first lane) or anti-SNAP-25 mAb (all other lanes) were performed. The immunoprecipitates were analyzed by SDS–PAGE and autoradiography. C: Quantitation of SNAP-25 phosphorylation. The amount of [^{32}P]SNAP-25 present in each condition shown in B was quantitated by phosphorimager analysis. The data are shown as the average ± S.D. for the triplicate samples. $*P<0.05$, $***P<0.001$ as compared to the control sample.

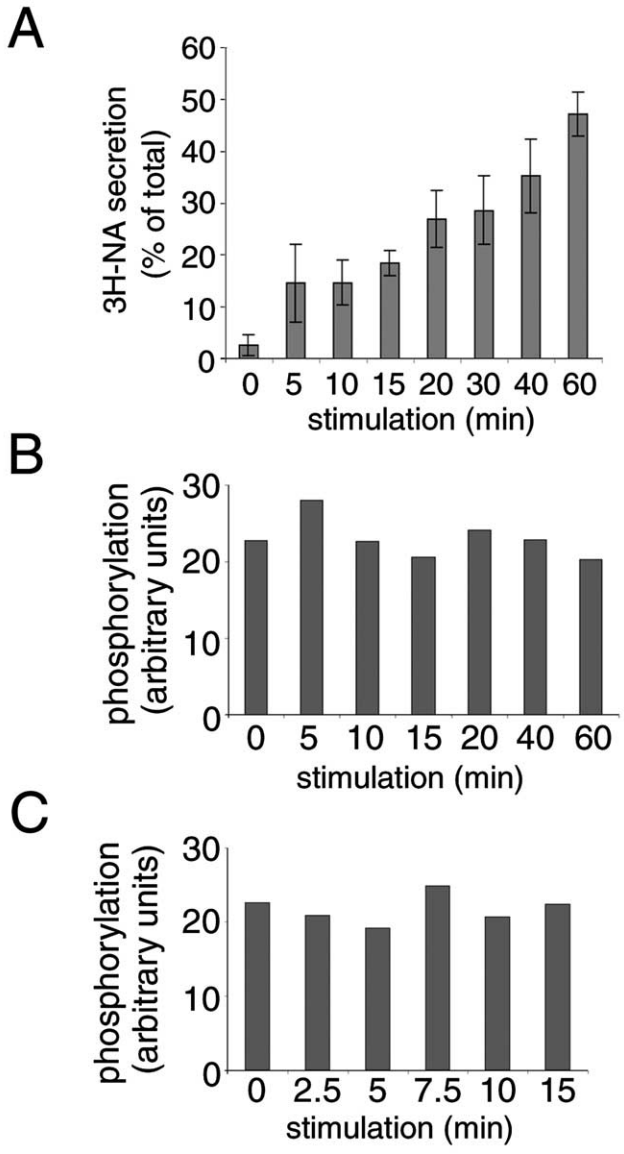

Fig. 4. Kinetics of regulated secretion and SNAP-25 phosphorylation in PC12 cells. A: Time course of regulated secretion. PC12 cells were loaded with [^{3}H]NA and exocytosis triggered with 59 mM KCl. Supernatants were harvested at various times and the cells were lysed. Secretion was calculated as a percentage of radioactivity present in the supernatant relative to the total amount of [^{3}H]NA taken up by the cells. B,C: SNAP-25 phosphorylation during PC12 cell secretion. PC12 cells were loaded with ^{32}P for 4 h prior to stimulation with KCl. At various times the cells were washed extensively in ice-cold buffer, lysed, and the amount of [^{32}P]SNAP-25 present in the cells was determined by immunoprecipitation, SDS–PAGE, and quantitative phosphorimager analysis.

creased SNAP-25 phosphorylation two-fold while PMA (alone or together with ionomycin) increased SNAP-25 phosphorylation four-fold (Fig. 3B,C). Most importantly, despite their stimulation of exocytosis, neither KCl, nicotine, nor ionomycin alone induced any measurable change in SNAP-25 phosphorylation.

Since these data were obtained 20 min after secretagogue administration, we explored the possibility that a transient 'burst' of phosphorylation occurred either prior to or subsequent to this time point. While we observed the expected time-dependent increase in neurotransmitter release from KCl-stimulated cells (up to ~50% release at 1 h, Fig. 4A), we did not detect any appreciable changes in SNAP-25 phosphorylation when assayed over the period of 60 min (Fig. 4B,C), demonstrating that efficient neurotransmitter release is independent of a measurable increase in SNAP-25 phosphorylation.

4. Discussion

Although Ser187 has been described as the unique target of PKC phosphorylation on SNAP-25 [5], we now have identified Thr138 as an additional PKC phosphorylation site of endogenous SNAP-25 in PC12 cells. Moreover, our identification of Thr138 as the exclusive in vivo target of PKA phosphorylation on SNAP-25 is in excellent agreement with in vitro data showing that this residue is phosphorylated by PKA [7]. Despite a previous report suggesting that induction of neurotransmitter release leads to SNAP-25 phosphorylation [5], we found no correlation between the ability of various secretagogues to induce vesicle exocytosis and their ability to induce SNAP-25 phosphorylation in PC12 cells. Indeed, PMA or forskolin treatment alone resulted in dramatic phosphorylation of SNAP-25 Ser187 and/or Thr138 without appreciable neurotransmitter release, while membrane depolarization using KCl or nicotine caused robust neurotransmitter release but had no detectable effect on SNAP-25 phosphorylation. We conclude therefore that the increase in SNAP-25 phosphorylation observed previously was a consequence of directly stimulating PKC by PMA and that physiological stimulation of neurotransmitter release from PC12 cells is not accompanied by dramatic changes in SNAP-25 phosphorylation.

During the preparation of this article Gonelle-Gispert et al. [13] reported that insulin secretion from pancreatic β-cells was also independent of SNAP-25 phosphorylation. This finding is consistent with our data showing a lack of correlation of SNAP-25 phosphorylation and exocytosis as well as studies showing that overexpression of the SNAP-25 Thr138/Ser187 double mutant did not interfere with KCl-induced exocytosis from PC12 cells (R. Hepp and P.A. Roche, data not shown). Together these data argue against an important role of SNAP-25 phosphorylation during the process of regulated exocytosis.

These data, along with those in the recent literature concerning Ser187 phosphorylation [14,15], show that massive SNAP-25 phosphorylation is not a prerequisite for efficient neurotransmitter release from PC12 cells. However, addition of nerve growth factor to PC12 cells can indeed lead to transient SNAP-25 phosphorylation over a period of days [9], although this process of differentiation is dependent upon cellular signaling and not necessarily exocytosis. Moreover, induction of long-term potentiation or epileptic-like seizures using bicuculline increases SNAP-25 phosphorylation in rat hippocampal slices [8]. In the case of long-term potentiation, increase of the postsynaptic response occurs after a tetanic stimulation of presynaptic neurons, while during drug-induced seizures hyperactivity and synchronization develop within the hippocampus neuronal network [16,17]. Thus, it is conceivable that sustained neuronal activity leads to kinase activation and SNAP-25 phosphorylation. The unambiguous identification of SNAP-25 Thr138 and Ser187 as targets for PKC and Thr138 as the target for PKA in vivo described in this study provides

the foundation for further studies aimed to clarify the role of phosphorylation for SNAP-25 function.

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