Inhibition of SNAP-25 Phosphorylation at Ser\textsuperscript{187} Is Involved in Chronic Morphine-induced Down-regulation of SNARE Complex Formation*

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Opiate abuse has been shown to cause adaptive changes in presynaptic release and protein phosphorylation-mediated synaptic plasticity, but the underlying mechanisms remain unclear. Neuronal SNARE proteins serve as important regulatory molecules underlying neural plasticity in view of their major role in the process of neurotransmitter release. In the present study, the expression of SNAP-25, a t-SNARE protein essential for vesicle release, was found to be dramatically regulated in hippocampus after chronic morphine treatment, which was visualized with two-dimensional gel electrophoresis. The spots of SNAP-25 in the gel were shifted along the dimension of isoelectric point, indicating a likely change of the post-transcriptional modification. Immunoblotting analysis with specific antibody to Ser\textsuperscript{187}, a protein kinase C (PKC) phosphorylation site of SNAP-25, revealed that the specific phosphorylation was correspondingly decreased, which was correlated with morphine-induced inhibition of PKC activity. Moreover, the level of ternary complex of SNARE proteins in either synaptosomes or PC12 cells was significantly reduced after chronic morphine treatment. This suggests a causal relationship between the inhibition of PKC-dependent SNAP-25 phosphorylation and the down-regulation of SNARE complex formation after chronic morphine treatment. Further analysis of SNARE complex formed by transfection of the wild-type or Ser\textsuperscript{187} mutants of SNAP-25 showed that only wild-type-formed complex was inhibited by morphine treatment. Thus, these results indicate that chronic morphine treatment inhibits phosphorylation of SNAP-25 at Ser\textsuperscript{187} and leads to a down-regulation of SNARE complex formation, which presents a potential molecular mechanism for the alteration of exocytotic process and neural plasticity during opiate abuse.

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Opine abuse causes extensive neural adaptive changes in the brain (1–3), which may be involved in a formation of aberrant learning (2). Accumulating evidences demonstrate that opiates significantly alter synaptic transmission and neural plasticity in the hippocampus, a center of learning and memory (4–8). Importantly, presynaptic neurotransmitter release, which can be modulated by phosphorylation, serves as a critical element in the regulation of synaptic transmission during opiate abuse. Many protein kinases involved in modulation of transmitter release and synaptic plasticity, such as cAMP-dependent kinase (PKA), protein kinase C (PKC), and Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII), also participate in cellular and synaptic adaptation mediating opiate dependence (3, 9). However, the protein substrates of these kinases involved in opiate abuse still need to be further elucidated.

Regulated membrane fusion of synaptic vesicles and subsequent transmitter release involves the assembly of ternary complexes from soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins (10). The ternary complex is formed by the plasma membrane proteins synaptosomal-associated protein of 25kDa (SNAP-25) and syntaxin, and vesicle-associated SNARE synaptobrevin 2 (synaptobrevin) (11). The SNARE proteins have been shown to be phosphorylated by various kinases (12), and accumulating evidences demonstrate that phosphorylation of SNAREs by various protein kinases plays an important role in modulating the molecular interactions between synaptic vesicles and the presynaptic membrane. For instance, phosphorylation of SNAP-25 by PKC regulates SNAP-25 localization (13, 14), affects interaction of SNAP-25 with syntaxin and synaptotagmin (15, 16), and potentiates the recruitment of vesicles to the plasma membrane (17, 18), whereas syntaxin1A phosphorylation by death-associated protein kinase (19) regulates its binding to Munc-18, a syntaxin1A-binding protein that regulates SNARE complex formation. In addition, many SNARE regulatory proteins have been shown to be phosphorylated in \textit{vitro}, which may alter the interactions of the proteins with their effectors and binding partners and hence modulate neurotrans-
mitter release (12). However, whether phosphorylation of SNARE proteins is modulated directly by opiate remains unclear, and the significance of phosphorylation of SNARE proteins in vivo during opiate abuse is still poorly understood.

In the current study, we showed that chronic morphine treatment inhibited the phosphorylation of SNAP-25, and this was attributable to the down-regulation of PKC activity. Moreover, we have shown that PKC-dependent phosphorylation participated in a down-regulation of SNAP-25 complex formation during morphine treatment. These results suggest that the phosphorylation of SNARE protein can be modulated in vivo, which may serve as a potential mechanism for the modulation of elementary steps of exocytotic process under opiate abuse.

EXPERIMENTAL PROCEDURES

Preparations of Mice Brain Tissue—Male C57BL/6J mice (20–22 g) were obtained from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). Mice were housed in groups and maintained on a 12-h light/dark cycle with food and water available ad libitum. All treatments were strictly in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were treated by repeated intraperitoneal injection of morphine (or saline in control) at an interval of 12 h, over 6 days (20 mg/kg on day 1 to 10 mg/kg/day on days 5 and 6) (20). The time mentioned in the results. Withdrawal was precipitated by injecting naloxone (1 mg/kg, subcutaneously) 2 h after the last morphine administration (20). Then the brains were removed, the hippocampus, prefrontal cortex, cerebellum, and brain stem were rapidly dissected, and the tissue was prepared for two-dimensional gel electrophoresis (2-DE) as mentioned below. The further subcellular fractions were prepared, respectively, according to the standard methods as described previously (21, 22). Briefly, brain samples were homogenized in ice-cold 0.32 M sucrose, 5 mM HEPES (pH 7.4), 0.1 mM EDTA, and described previously (21, 22). Briefly, brain samples were homogenized in ice-cold 2-DE sample buffer (4°C), and the supernatants were spun at 10,000 × g for 20 min in a microcentrifuge at 4°C. The pellets constituted the crude synaptosomal fraction (P2). Then the fractions were resuspended in 500 μl of 1× P buffer (5.4 mM KCl, 0.8 mM MgSO₄, 5.5 mM glucose, 50 mM HEPES, 130 mM choline chloride, 1 mM bovine serum albumin, and 0.01% CHAPS). Synaptosomes were then solubilized with 0.5 ml of buffer (150 mM NaCl, 10 mM HEPES, and 1.5% CHAPS, pH 7.4), stirred at 4°C and 10 min; 4°C–4°C, for 1 h and then centrifuged at 12,000 × g for 1 h. After a protein concentration of ~0.5 mg/ml that was determined by Bradford methods (23), this suspension was used in Western blot analysis as described below.

Two-dimensional Gel Electrophoresis (2-DE) for Protein Identification—Brain samples were homogenized in ice-cold 2-DE sample buffer (4°C), and the supernatants were spun at 10,000 × g for 20 min in a microcentrifuge at 4°C, and the supernatants were collected. Two-DE was performed according to the previous studies (24, 25). 100 μg of total protein for analytical 2-DE gel and 1 mg for preparative gel were loaded on nonlinear immobilized pH gradient (IPG) strips (NL pH 3–10, Amersham Biosciences), respectively, and hydrated in hydration buffer (8 M urea, 2% CHAPS, 18 mM dithiothreitol, 0.5% IPG buffer, bromphenol blue trace) for 10 h. Isoelectric focusing was performed, at 20°C, for 2 h at 500 V, for 1 h more at 1,000 V, and then for 12 h at 8,000 V in an IPGphor apparatus (Amersham Biosciences) with a limiting current of 70 μA per strip. Proteins were resolved on a second dimension. The strips were incubated in 50 mM dithioerythritol buffer (6 μl urea, 2% SDS, 30% glycerol, 50 mM Tris-HCl, pH 6.8) for 15 min to reduce S–S bonds. The 12-HG bands were subsequently blocked for 15 min with the same solution except that the dithioerythritol was replaced by iodoacetamide (2.5%) and a trace of bromphenol blue. Equilibrated strips were inserted onto 12.5% SDS–PAGE gels. The gels were run under constant current 10 mA/gel for 0.5 h and 30 mA/gel for 3.5 h at 10°C (Bio-Rad). Analytical gels were visualized by silver staining or were electroblotted onto nitrocellulose and then incubated with the monoclonal antibody to SNAP-25 as described below. Protein spots excised from 2-DE gels in preparative gel were identified by Western blotting. Western blotting was performed as described above or subjected to in-gel digestion with trypsin, and the resulting peptides were characterized by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) and data base searching.

RESULTS

Regulation of SNAP-25 following Chronic Morphine Treatment—To test the potential changes of protein expression in RPMI 1640 media (Invitrogen) supplemented with 5% horse serum/10% fetal bovine serum/50 units/ml penicillin, and 50 μg/ml streptomycin. Cells (10⁶) were incubated in a humidified atmosphere of 5% CO₂. After chronic treatment with morphine (0.01–10 μM) or saline for the indicated time (1–24 h), the cells were lysed and analyzed by Western blot analysis as described below.

Transfection—Enhanced green fluorescent protein (EGFP)-SNAP-25 (Wild-type), EGFP-SNAP-25ΔE (S187E), and EGFP-SNAP-25ΔA (S187A) were kindly provided by Dr. Masakazu Kataoka (Dept. of Environmental Science and Technology, Shinshu University, Nagano, Japan) (14). Transfection of PC12 cells with eukaryotic expression plasmids was performed by using LipofectAMINE 2000 (Invitrogen) as previously described (26). After 24 h of transfection, the cells were treated with morphine as mentioned above and analyzed by Western blotting.

Electrophoretic Procedure and Western Blot Analysis—The samples (20 μg of protein) from brain tissue or cultured cells of control and morphine-treated groups were incubated at 100°C or 22°C for 5 min, respectively, before electrophoresis. Then the samples were loaded and subjected to a discontinuous gel, with the upper two-thirds being 8% and the lower one-third being 15% acrylamide, respectively, and were electroblotted onto nitrocellulose membrane by using a minigel and mini transblot apparatus (Bio-Rad). The membranes were blocked in blocking buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, and 0.1% Tween 20, 15% nonfat milk) at room temperature for 1 h. Then the membranes were incubated with antibodies of mouse anti-SNAP-25 (1:10,000) or mouse anti-(20 μg/ml) on day 1 (1:10,000), or mouse anti-GFP (1:10,000) from Synaptic Systems (Göttingen, Germany), or rabbit anti-phosphorylated SNAP-25 (1:1,000; a kind gift from Masami Takahashi, Kitasato University, Kanagawa, Japan), or mouse anti-GFP (1:1000; Roche Applied Science), respectively, overnight at 4°C. The membranes were washed and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody for 1 h at room temperature. Finally, the blots were visualized with enhanced chemiluminescence (Amerham Biosciences). For the quantification of the Western blot data, the developed films were scanned, the immunoreactive bands were digitized, and the densitometry was performed using Scion Image for Windows (Scion, Frederick, MD). The re-electrophoresis in a second dimension for detecting SNARE complexes was carried out as described previously (27). In brief, after the first dimension, the whole lane containing the separated proteins was excised, soaked for 20 min in 10% (v/v) acetic acid and 25% (v/v) isopropanol, washed with H₂O for 5 min, and then incubated for 20 min in SDS sample buffer. After heating for 5 min at 100°C, the strip was mounted on top of a 15% gel, re-electrophoresed, and analyzed by Western blotting as mentioned above.

Protein Kinase Activity Assay—PKC and PKA activity was determined essentially according to the method described previously (28). Animal was rapidly decapitated, and hippocampus was dissected rapidly and the hippocampal synaptosomal fraction was isolated as described above. The synaptosomes were resuspended in 50 mM Tris-HCl, 8 mM MgCl₂, 10 mM NaCl, 1.5% CHAPS, pH 7.4, and further centrifuged at 12,000 × g for 1 h. After a protein concentration of ~0.5 mg/ml that was determined by Bradford methods (23), this suspension was used in Western blot analysis as described below.
the hippocampus during opiate abuse, mice were injected with morphine in an increasing dose for 6 days, a procedure known to produce significant morphine dependence (20), and then the proteome analysis of hippocampal protein expression was conducted. The hippocampal extracts were separated by high resolution 2-DE, and SNAP-25, a t-SNARE protein located within presynaptic terminals and essential for vesicle release (29, 30), was identified with MALDI-TOF-MS followed by data base searching and further confirmed by immunoblotting with antibody to SNAP-25 (Fig. 1, A and B). Interestingly, the spots of SNAP-25 in the lower isoelectric points (pIs) were dramatically redistributed to higher pI after the treatment, indicating a likely change of the post-transcriptional modification (Fig. 1A). To further investigate the expression pattern of SNAP-25, the 2-DE gel was electroblotted onto nitrocellulose membrane and visualized with antibody to SNAP-25. The expression of phosphorylated SNAP-25 in the 2-DE membrane was also detected with a specific antibody to Ser187 (13, 17), a specific phosphorylated SNAP-25 in the 2-DE membrane was also detected visualized with antibody to SNAP-25. The expression of phosphorylated SNAP-25 in the 2-DE membrane was also detected with a specific antibody to Ser187 (13, 17), a specific phosphorylation site in the C-terminal (15). The result of Fig. 1C showed that, following chronic morphine treatment, the relative intensity of the immunoblotting spots visualized with antibody to SNAP-25 was considerably altered. Concretely, the labeling of spots on the left (low pI) became weaker, whereas the spots on the right (high pI) were more intensely labeled (Fig. 1C). No significant change was observed in the total level of SNAP-25 expression. The phosphorylated SNAP-25 mainly distributed in the spots of left part and was significantly decreased in morphine-treated group (Fig. 1D), which was consistent with the redistribution of the spots visualized with antibody to SNAP-25. The expression pattern of SNAP-25 on the blots of the 2-DE was similar to that shown in the previous study in which spots were demonstrated to represent different states of phosphorylation of SNAP-25 by PKC but not by casein kinase II or CaMKII (24). We also detected the expression pattern of syntaxin1A or synaptobrevin, other SNARE proteins, by using their specific antibodies, and no significant changes were observed after chronic morphine treatment under the same conditions (data not shown).

Inhibition of PKC Activity and PKC-dependent Phosphorylation of SNAP-25—To examine the regulation of SNAP-25 at synapses, the synaptosomal fraction of the hippocampus was prepared and the protein expression of total and phosphorylated SNAP-25 was detected, respectively. In agreement with the results of 2-DE gel, the level of phosphorylated SNAP-25 was decreased after chronic morphine treatment, whereas the total SNAP-25 expression was unchanged (Fig. 2A). Moreover, when the morphine-treated mice were injected with naloxone (1 mg/kg), a nonspecific opioid receptor antagonist documented to lead to morphine withdrawal (20), 2 h after the last morphine administration, the decreased level of phosphorylated SNAP-25 was restored to the normal level (Fig. 2B). We also detected expression of phosphorylated SNAP-25 in synaptosomal fraction of other brain regions under the same condition, and a similar decrease was observed in the brain stem but not in the prefrontal cortex and the cerebellum (Fig. 2, C and D), suggesting differential sensitivity of brain regions to chronic use of opiate.

It is known that SNAP-25 is phosphorylated by PKC (15), and PKC expression in hippocampus decreases after chronic morphine treatment in the brain (31, 32), but how the activity of PKC is regulated in the hippocampus by morphine remains unknown. We found that the PKC activity in hippocampal synaptosomes was significantly decreased 2 h after the last morphine administration, and then recovered 8 h later (Fig. 3B), whereas a significant increase in PKA activity was ob-

![FIG. 1. Regulation of SNAP-25 in chronic morphine treated mice. A, the proteome analysis of the hippocampal protein expression was conducted in the mice following treatment of morphine in an increasing dose (from 20 mg/kg to 100 mg/kg, intraperitoneal) for 6 days. The high resolution two-dimensional electrophoresis (2-DE) gel was visualized with silver staining. Arrows indicate the altered proteins. A relative decrease in the optical density of the left spots (lower isoelectric point) and an increase in that of the right spots (higher isoelectric point) were observed after morphine treatment. These spots were identified as SNAP-25 by using MALDI-TOF-MS and data base searching. B, these spots in the two-dimensional gel were also excised, respectively, and further confirmed by immunoblotting with antibody to SNAP-25. Spots 1–10 as indicated by arrows in the two-dimensional gel (the upper panel) were excised for immunoblotting, and spots 4, 5, 6, and 7 corresponding to SNAP-25 on the gel were identified (the lower panel). The data were pooled from two independent experiments. The slight labeling of spot 8 might be caused by the contamination of spots 4 or 5, because the density was much lower than other spots, and the position of molecular weight was higher. TL, total lysates; Sal, saline treatment; Mor, morphine treatment. C, the optical density of the spots visualized with antibody to SNAP-25 on the left decreased, while that on the right increased following chronic morphine treatment. The relative density of SNAP-25 spots on the left and right is expressed as the percentage of the summation of the measures for all spots in the right panel. Data are presented as mean ± S.E. values of the results obtained in three separate experiments. D, the phosphorylated SNAP-25 was mainly distributed in the spots on the left and was significantly decreased in the phosphorylated group. Group data (mean ± S.E.) of SNAP-25 and phosphorylated-SNAP-25 in C and D for hippocampal homogenates of morphine-treated mice were expressed as percentages of that of saline-treated rats in the right panel (mean ± S.E. *) *p < 0.01, Student’s t test; as compared with saline group. Sal, saline treatment; Mor, morphine treatment.](516x737)
D* served after chronic morphine treatment (Fig. 3, C). The time course of changes in PKC activity was parallel with that in levels of phosphorylated SNAP-25 (Fig. 3, F and H). We also detected the effect of acute morphine treatment (10–100 mg/kg) on PKC activity and SNAP-25 phosphorylation, and no obvious change was observed (Fig. 3, A, C, E, and G).

**Down-regulation of SNARE Ternary Complex after Chronic Morphine Treatment**—Because SNAP-25 is a key component of the synaptic vesicle docking/fusion machinery that binds with synaptobrevin and syntaxin to form the SNARE complex, we want to know whether the abundance of the ternary complex of SNARE proteins was also altered after chronic exposure to morphine. The ternary complex is highly stable, such that it resists denaturation by SDS and is disrupted only upon boiling of sample (33). Therefore, it is possible to detect the signal of SNARE complex by comparing boiled and nonboiled samples. Fig. 4A shows that a high molecular mass band of ternary complex in ∼100 kDa was detected in nonboiled samples. The band disappeared after boiling the samples, which was accompanied with a corresponding increase in the level of monomeric SNAP-25. The band at ∼100 kDa was also detected using antibodies to syntaxin or synaptobrevin (Fig. 4A). Moreover, all the three proteins could be detected when the gel in the position of the band was excised, boiled, and reloaded (data not shown), indicating that the band containing all three proteins is one of the forms of ternary complex. As shown in Fig. 4B, after chronic morphine treatment, a significant decrease in the high molecular mass band at 100 kDa and an increase of
monomeric SNAP-25 band was observed, whereas the total expression level of SNAP-25 remained unchanged (Fig. 4C). Interestingly, naloxone-initiated withdrawal could restore morphine-altered SNAP-25 to a high level (Fig. 4B), suggesting that the change of the complex is correlated to the modulation of morphine level in mice. Similar results were also obtained when the antibodies to syntaxin1A and synaptobrevin was applied, respectively (data not shown).

SDS-resistant complexes contain large forms with distinct molecular weight (27), and anti-SNAP-25 antibody may not recognize all the forms of these complexes in the hippocampal samples. Thus it is essential to examine whether the other high molecular weight bands are also altered by chronic morphine treatment. In the present study, the gel lane in saline and morphine groups was excised, respectively, after electrophoresis, heated to 100 °C to disrupt the complexes, and re-electrophoresed under identical conditions in a second dimension. Fig. 4D shows that the SDS-resistant complexes were visualized with any one of the three antibodies. After chronic morphine treatment, the expression level of complexes was significantly down-regulated, which was accompanied with an up-regulation of monomeric proteins, as compared with the saline group (Fig. 4D). This was consistent with previous observations.

**Regulation of SNARE Complex Formation by PKC-dependent Phosphorylation of SNAP-25**—To clarify the possible involvement of PKC-dependent SNAP-25 phosphorylation in the regulation of SNARE complex formation by chronic morphine treatment, PC12 cells were used as a cellular model, in which both opioid receptor and δ opioid receptor were expressed (34–37), to further detect the effect of morphine. As shown in Fig. 5A, the phosphorylation of SNAP-25 at Ser187 in PC12 cells was markedly reduced in a dose-dependent manner (Fig. 5A), and the decrease appeared 1 h after morphine incubation (Fig. 5B).

In addition to the SNAP-25 phosphorylation, the high molecular mass band at 100 kDa was also detected in PC12 cells using antibodies to SNAP-25, syntaxin1A, and synaptobrevin, respectively. Consistent with the data in hippocampal fraction, the band at 100 kDa was recognized and could be abolished by boiling samples (Fig. 6A). After morphine treatment, the high molecular mass band was significantly down-regulated in a dose- and time-dependent manner (Fig. 6B and C). However, the total expression level of SNAP-25 in PC12 cells remained unchanged after morphine treatment in the indicated time and dose (Fig. 5, A and B).

The next question we asked was whether the phosphorylation of SNAP-25 was implicated in the regulation of SNARE
complex formation. Wild-type (WT) and mutant constructs of SNAP-25 (S187E and S187A), in which Ser^187 was replaced by Glu or Ala, were fused with EGFP at the N terminus, expressed in PC12 cells, and analyzed with an anti-GFP antibody. S187E was used to mimic the constitutively phosphorylated state, whereas S187A to mimic the nonphosphorylated form of serine. As shown in Fig. 7, two high molecular mass bands at ~100 kDa and ~130 kDa were recognized by anti-GFP antibody after transfection of SNAP-25 in PC12 cells (Fig. 7A), but only the ~130-kDa band could resist the high temperature incubation up to 95 °C (Fig. 7B). This suggests that the ~130-kDa band is the ternary complex formed by EGFP-tagged SNAP-25 transfection into PC12 cells. Moreover, S187E expression potentiated the level of the formed ternary complex, whereas S187A expression attenuated it (Fig. 7A). Twenty-four hours after incubation of PC12 cells with morphine, the expression level of the ternary complex at ~130 kDa containing WT was attenuated, whereas the expression patterns of either S187E or S187A groups were not changed (Fig. 7C and D). This indicates that SNAP-25 phosphorylation at Ser^187 is involved in the down-regulation of SNAP-25 complex formation following chronic morphine treatment.

**Fig. 6. Down-regulation of SNAP-25-involved SNARE complex formation in PC12 cells after chronic morphine treatment.** A, the high molecular weight band at ~100 kDa, which represented SNAP-25 complex, could be detected in PC12 cells with antibodies to SNAP-25, syntaxin1A, and synaptobrevin respectively, and could be abolished by boiling samples. B, chronic treatment of PC12 cells with morphine 0.01–10 μM for 12 h decreased the expression of SNAP-25 at ~100 kDa in a dose-dependent manner. C, chronic preincubation with morphine 1 μM for 1–12 h attenuated the SNAP-25 expression at ~100 kDa in a time-dependent manner. The immunoblots in B and C were representative of three independent experiments.

**Fig. 7. Phosphorylation of SNAP-25 participated in the regulation of SNARE complex formation during chronic morphine treatment.** A, high molecular mass band at ~130 kDa, which represented SNAP-25 complex, could be observed in PC12 cells transfected with EGFP-tagged SNAP-25 but not in untransfected cells or hippocampal synaptosomes (Hip) after immunoblotting with anti-GFP antibody. The complex signal at ~130 kDa was stronger in the group transfected with S187E and was weaker with S187A than that with WT. Immunoblotting of the boilded sample detected with anti-GFP antibody shows the expression of total transfected EGFP-SNAP-25. B, the complex signal at ~130 kDa could not be abolished until the sample was treated above 95 °C. C, after morphine treatment for 24 h, the complex signal at ~130 kDa was attenuated in the WT group, whereas no significant change could be observed in either S187E or S187A groups. Immunoblots were representative of three independent experiments. D, the complex signal of SNARE complex was quantitated, and the density in the morphine groups was expressed as the percentage of that in the saline group transfected with WT (mean ± S.D.). *, p < 0.05 Student’s t test, as compared with the saline group. Sal, saline treatment; Mor, morphine treatment.

**DISCUSSION**

Studies on opiate dependence have been focused on protein phosphorylation-mediated synaptic plasticity, which are generally thought to be part of the neural basis of an addictive state (38, 39). Neuronal SNARE proteins serve as important regulatory molecules underlying neural plasticity (24, 40, 41) in view of their major role in the process of neurotransmitter...
release. In this report we demonstrated that phosphorylation of SNAP-25 in PC12 cells (13, 14). Further electrophysiological study shows how the SNARE complex and the related step of complex formation was up-regulated after PMA treatment, and indicated that the PKC pathway participated in the modulation of SNARE complex formation, which may be involved in PKC-mediated exocytotic process during opiate abuse.

The molecular mechanism of PKC-mediated vesicle exocytosis remains an open question. It has been shown that PKC mediates secretory vesicle translocation to the plasma membrane (18, 47). Lonart and Südhof (48) also propose a working model that SNARE complex assembly prior to fusion pore opening is up-regulated by PKC and then sets the readily pool of vesicles. Our unpublished data show that SNARE complex formation was up-regulated after PMA treatment, and PMA-stimulated up-regulation of complex formation was inhibited by PKC blocker, indicating that the PKC pathway participated, at least in part, in SNARE complex formation. However, how the SNARE complex and the related step of membrane fusion are regulated by the PKC pathway is still elusive.

Noticeably, PKC can mediate the localization of SNAP-25 in PC12 cells (13, 14). Further electrophysiological study shows that PKC-dependent phosphorylation of SNAP-25 at Ser187 potentiates the vesicle recruitment (17), which indicates a critical role of SNAP-25 phosphorylation in the refilling of vesicle pools rather than in the fusion of vesicles from the readily releasable pool (17, 49). With biochemical methods, the present study demonstrated that SNAP-25 phosphorylation at Ser187 participated in the modulation of SNARE complex formation, which may lead to several considerations about the phosphorylation-mediated regulation of SNAP-25 complex and its possible effect on vesicle recruitment. On the one hand, previous biochemical study demonstrates that phosphorylation of SNAP-25 accelerated the dissociation of SNAP-25 from syntxin in vitro (15). It is possible that phosphorylation of SNAP-25 speeds up disassembly of nonproductive binary complexes, involving two molecules of syntxin and one SNAP-25, thereby favoring the formation of productive ternary complexes by replacing one syntxin with synaptobrevin (50). This possibility was also mentioned in the previous study (17). On the other hand, SNAP-25 complex formation regulated by SNAP-25 phosphorylation may function as fusion machinery localized in the membrane of synaptic vesicles participating in vesicle refilling and translocation to the plasma membrane (51), because SNAP-25 complex can be assembled and disassembled in vesicle membranes (27) and governs the probability of synaptic vesicles (52). Although the evidence to support these possibilities is not adequate so far, these hypotheses merit future investigation of the mechanism of opiate action.

In summary, the present study revealed a decrease of SNAP-25 phosphorylation in chronically morphine-treated hippocampal synaptosomes, which is involved in consequence down-regulation of SNAP-25 complex formation. The alteration in SNAP-25 phosphorylation and SNAP-25 complex formation may serve as a potential molecular mechanism in the modulation of neurotransmission and synaptic plasticity under opiate abuse, which may be associated with the development of aberrant learning and memory induced by abused drug.

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REFERENCES

30. Sorensen, J. B., Nagy, G., Varoqueaux, F., Nehring, R. B., Brose, N., Wilson,

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