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Two Distinct Mechanisms Underlie the Stimulation of Neurotransmitter Release by Phorbol Esters in Clonal Rat Pheochromocytoma PC12 Cells[1]

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Phorbol ester treatment induces the phosphorylation of SNAP-25 at Ser[187] and the potentiation of Ca^{2+}-induced dopamine (DA) and acetylcholine (Ach) release from PC12 cells. In order to evaluate the functional consequences of phosphorylation, quantitative analysis was carried out using an anti-phosphopeptide antibody that specifically recognizes SNAP-25 phosphorylated at Ser[187]. DA and ACh release, assayed in low-K^+ as well as high-K^+ solution, increased by treating the cells with phorbol-12-myristate-13-acetate (PMA); however, the stimulation of high-K^+-dependent release occurred at lower concentrations and with shorter exposures to PMA than that of the basal release in low-K^+-solution. The PMA-induced phosphorylation of SNAP-25 did not correlate with the potentiation of high-K^+-dependent neurotransmitter release. The potentiation of high-K^+-dependent DA release by phorbol 12,13-diacetate (PDA), a water soluble phorbol ester, almost completely disappeared within 1 min after washing PDA in the presence of okadaic acid, conditions under which the phosphorylation of SNAP-25 persisted for at least 15 min. PMA-induced phosphorylation of SNAP-25 was inhibited by staurosporine, however, the potentiation of high-K^+-dependent DA release was suppressed only partially. These results indicate that protein kinase activation does not account for a large fraction of the phorbol ester-induced potentiation of depolarization-dependent neurotransmitter release from PC12 cells.

Key words: neurotransmitter release, phorbol ester, phosphorylation, PKC, SNAP-25.

Synaptic transmission is mediated by neurotransmitters released from synaptic nerve terminals. Neurotransmitters are stored in synaptic vesicles and released into the synaptic cleft by exocytosis of synaptic vesicle contents, which involves docking and fusion of the synaptic vesicle membrane with the presynaptic plasma membrane (*1*). Recent studies have revealed that so-called SNARE proteins, including VAMP-2 (also called synaptobrevin-2) in the synaptic vesicle membrane, and SNAP-25 and syntaxin in the plasma membrane, play an essential role in the exocytosis of synaptic vesicles (*2*). Clostridial neurotoxins, such as botulinum neurotoxins and tetanus toxin, are Zn^{2+}-dependent proteases that cleave SNARE proteins at specific sites, and inhibit neurotransmitter and hormone release from neurons and endocrine cells (*3*). SNARE proteins have coiled-coil motifs in their structures, and protein–protein association mediated by these motifs is believed to be indispensable for exocytosis (*4*). Recently, membrane fusion was shown to occur between liposomes containing VAMP-2 and those containing SNAP-25 and syntaxin (*5*), although the fusion rate of the reconstituted proteoliposomes was quite low. Many additional proteins are believed to be involved in neurotransmitter release from synaptic nerve terminals, however, their precise roles remain obscure.

Modulation of synaptic transmission underlies the plasticity of neuronal networks, which is believed to constitute the molecular and cellular basis of learning and memory (*6*). Both pre- and post-synaptic mechanisms are involved in the regulation of synaptic transmission, but their contributions vary among different synapses. The regulation of neurotransmitter release is the major presynaptic mechanism and several different pathways have been suggested in various neuronal preparations. In some cases, ion channels that control Ca^{2+} influx are targets for regulation, and

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Abbreviations: ACh, acetylcholine; BoNT/A and BoNT/E, botulinum type A and E neurotoxin; DA, dopamine; KLH, keyhole limpet hemocyanin; PDA, phorbol 12,13-diacetate; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; SNAP-25, synaptosomal-associated protein of 25 kDa; SNAREs, SNAP receptors; TBS, Tris-buffered saline.

in other cases, a modulation of the exocytotic machinery can potentiate or suppress neurotransmitter release from nerve terminals (*7*, *8*). Various protein kinases have been implicated in presynaptic regulation in a wide range of neuronal preparations (*9*). Protein kinase C (PKC) is a Ca^{2+}/phospholipid-dependent protein kinase that participates in the regulation of many cellular functions (*10*). Some isoforms of PKC are localized in presynaptic terminals and may thus regulate neurotransmitter release (*11*), however, the relevant protein substrates of PKC have not yet been identified.

A rat clonal pheochromocytoma PC12 cell line has certain properties characteristic of immature rat adrenal chromaffin cells (*12*). These cells synthesize catecholamines, mostly dopamine (DA) and acetylcholine (ACh), and store them in large dense-core vesicles and small synaptic vesicles, respectively. PC12 cells can then release these neurotransmitters by Ca^{2+}-dependent exocytosis. Treating the cells with phorbol esters, activators of PKC, greatly potentiates Ca^{2+}-dependent catecholamine and ACh release (*13*). Previously, we showed that SNAP-25 is phosphorylated on Ser^{187} after phorbol-12-myristate-13-acetate (PMA) treatment of PC12 cells, and that the association of SNAP-25 and syntaxin is suppressed under phosphorylation conditions (*14*). However, it is not known whether SNAP-25 phosphorylation is essential for the PMA-dependent regulation of neurotransmitter release. In the present study, we generated an antibody that specifically recognizes phosphorylated SNAP-25 and examined the correlation between phosphorylation and the enhancement of neurotransmitter release by quantitative analysis. We found that phorbol ester exerts its action through two distinct mechanisms, one phosphorylation-dependent and the other phosphorylation-independent.

EXPERIMENTAL PROCEDURES

Materials—Reagents and chemicals were obtained as follows: phorbol 12,13-diacetate (PDA), phorbol-12-myristate-13-acetate (PMA), Sigma (St. Louis, MO); staurosporine, Alomone labs (Jerusalem, Israel); okadaic acid sodium salt and 1-norokdaone, Calbiochem (La Jolla, CA); Epoxy-activated Sepharose 6B and biotinylated anti rabbit IgG, Amersham Pharmacia Biotech (Uppsala, Sweden); rabbit anti mouse IgG, Zymed Lab. (San Francisco, CA); rhodamine-goat anti mouse IgG, ICN Pharmaceuticals (Costa Mesa, CA); fluorescein-avidin, Vector Laboratories (Burlingame, CA); Imject Maleimide Activated Keyhole Limpet Hemocyanin, Pierce Chemical Company (Rockford, IL), Freund's complete and incomplete adjuvant (FCA and FIA, respectively), Becton Dickinson and Company. (Franklin Lakes, NJ); all other reagents were purchased from Sigma (St. Louis, MO), Wako (Osaka) or Bio-Rad Lab. (Hercules, CA).

Antibodies—The monoclonal anti SNAP-25 antibody (mAb BR05) (*15*) was prepared as described previously. The anti phosphorylated SNAP-25 antibody was raised to a peptide having the sequence (C)MEKADS(Pi)NKTRI, equivalent to SNAP-25 residues 182–192, which includes the phospho-serine at position 187. Ten milligrams of the synthetic phosphopeptide were conjugated with 5 mg of Keyhole Limpet Hemocyanin (KLH) according to the procedure recommended by the supplier. KLH-conjugated antigen peptide (0.25 mg peptide per rabbit) emulsified in FCA was injected subcutaneously at multiple sites on the backs of rabbits (New Zealand White). After 1 month, the same amount of antigen emulsified in FIA was injected as above. Immunization with FIA was repeated twice at intervals of 1 month. Whole blood was collected 10 days after the final immunization.

Purification of Anti Phosphorylated SNAP-25 Antibody—The antigen phosphopeptide and unphosphorylated peptide (5.4 mg each) were conjugated with Epoxy-activated-Sepharose 6B (0.66 g) according to the manufacturer's protocol. The phosphopeptide-conjugated resin was mixed with the antiserum and washed extensively in succession with 0.1 M Tris-HCl (pH 8.0), 0.5 M NaCl and 0.1 M Tris-HCl (pH 8.0), and 0.01 M Tris-HCl (pH 8.0). The bound antibody was eluted with 0.1 M glycine (pH 2.5) and immediately neutralized with Tris-HCl. The purified antibody was further mixed with the unphosphorylated peptide-conjugated Sepharose 6B and the antibody not bound to the resin was used as a specific antibody to phosphorylated SNAP-25 (pAb SN25Pi) in the present study. BSA and sodium azide at final concentrations of 1 mg/ml and 0.01M, respectively, were added to the purified antibody, which was then stored at −30°C until use.

PC12 Cell Culture—PC12-C3 cells (*16*), which store large amounts of ACh and DA, were used throughout the present study. The cells were maintained in Dulbecco's modified Eagle's medium (GIBCO BRL, Life Technologies, Rockville, MD) containing 5% (v/v) precolostrum newborn calf serum (Mitsubishi Chemical, Tokyo) and 5% (v/v) heat-inactivated horse serum (Gibco BRL) at 37°C in a humidified atmosphere of 5% CO_2, 95% air. Two days before experiments, 10^6 PC12-C3 cells were plated on 35-mm polyethyleniminecoated plastic culture dishes.

Immunofluorescence Microscopy of PC12-C3 Cells—PC12-C3 cells cultured on polyethyleneimine-coated glass slides (Nalge Nunc International) were fixed with 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate (pH 7.2) for 30 min at room temperature, and permeabilized with 0.1% (v/v) Triton X-100, 5% (v/v) goat serum in PBS. The cells were incubated with primary antibodies in the same solution overnight at 4°C. After washing with PBS, they were incubated with biotinylated anti–rabbit IgG and rhodamine-goat anti–mouse IgG, followed by fluorescein-avidin. Specimens were mounted with the anti–fader reagent ProLong (Molecular Probes), and observed under a laser scanning confocal microscope (MRC-1024, Bio-Rad Lab., Hercules, CA) equipped with an Axioplan microscope (Zeiss).

Neurotransmitter Release Assay—Cells were preincubated at 37°C for 17 min in 1.0 ml of low-K^+ solution (140 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11 mM glucose, and 15 mM HEPES-Tris, pH 7.4). The cells were rinsed twice with 1 ml of the low-K^+ solution, and then the medium was replaced with 0.6 ml of low-K^+ solution and 0.6 ml of the high-K^+ solution (115 mM NaCl, 30 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11 mM glucose, 15 mM HEPES-Tris, pH 7.4). For the determination of DA and ACh, the solution was immediately transferred at the end of the incubation periods to a microtube containing 150 μl of 1 M percholic acid (PCA). Cells were sonicated on ice with 500 μl chilled 0.2 M PCA and 0.1 mM EDTA. The samples were centrifuged at 15,000 rpm for 5 min at 4°C and the supernatant

was stored at −80°C for assay of ACh and DA.

DA Assay by HPLC—Released and cellular DA were assayed by HPLC using a reverse-phase column (TSKgel ODS-80TM, 4.6 mm × 10 cm, Tosoh, Tokyo) and an electrochemical detector (ECD) system as described (*16*). The mobile phase was composed of 85 mM NaH_2PO_4 buffer (pH 3.7) containing 15% (v/v) methanol, 20 μM EDTA, and 2.5 mM sodium 1-octanesulfonate (Nakarai Tesque, Kyoto). The column was maintained at 34°C and the flow rate was 1 ml/min. The applied potential at the working electrode was at +700 mV against Ag/AgCl and the detector range was 2 nA full scale. Essentially, the only CA detected in PC12-C3 cells was DA. The amount of DA released is expressed as a percentage of the total cellular DA content.

ACh Assay by HPLC—To determine ACh content, the samples were neutralized with K_2CO_3 and left for 30 min on ice. The samples were centrifuged at 15,000 rpm for 5 min at 4°C and the supernatants (190 μl) were filtered through 0.22-μm pore membrane filters (Ultrafree-C3-LG, Millipore) after the addition of 10 μl of 100 mM EDTA. Aliquots (10–25 μl) were analyzed by HPLC with the ECD system. The HPLC-ECD procedure was developed originally by Potter *et al.* (*17*) and improved by Asano *et al.* (*18*). The system consists of a reverse-phase column (Eicompak AC-GEL, 6 mm × 15 cm, Eicom), an immobilized ACh esterase and choline oxidase column (AC-Enzympak, 4 mm × 5 mm, Eicom), and an ECD (ECD-300, Eicom) with a platinum electrode. The mobile phase was composed of 0.1 M Na_2HPO_4 buffer (pH 8.5) containing 20 μM EDTA, 65 mg/liter tetramethylammonium chloride (Sigma), and 200 mg/liter sodium 1-decanesulfonate (Tokyo Kasei Kogyo, Tokyo). The columns were maintained at 33°C with a column oven and eluted at a flow rate of 1 ml/min. The applied potential at the working electrode was +450 mV *versus* the Ag/AgCl reference electrode and the detector range at full scale was 0.1 nA. The amount of ACh released is expressed as a percentage of total cellular ACh content.

Immnoblotting—SDS-PAGE was performed on 12.5% acrylamide gel according to Laemmli (*19*). Proteins were electrophoretically transferred onto polyvinylidene difluoride membrane (Immobilon-P, Millipore Corporation, Bedford, MA) with a semi-dry transblotting apparatus. The membranes were blocked in 10% nonfat milk in Tris-buffered saline containing 0.05% Tween 20 (TTBS) and incubated for 1 h with the antibodies, then treated with peroxidase-labeled anti mouse or anti rabbit IgG (DS grade, Zymed Laboratories) for 1 h at room temperature. After the reaction, the membranes were washed with TTBS, and the reactivity was detected with an enhanced chemiluminescence kit (Amersham). Quantification of the immunoreactive bands was performed by scanning densitometry of the X-ray film using ATTO densitogram ver 3.0 (ATTO, Tokyo), or by a luminescent image analyzer with an electronically cooled CCD camera system (LAS-1000, Fuji Photo Film, Tokyo).

Protein Amount Determination—Protein was measured by the method of Bradford using bovine gamma-globulin as a standard (*20*).

RESULTS

Generation of an Antibody that Specifically Recognizes SNAP-25 Phosphorylated by PKC—Previously, we reported that a Ser residue located between the cleavage sites of BoNT/A and BoNT/E is specifically phosphorylated in PC12 cells after PMA treatment (*14*). Since Ser^{187} is the only Ser residue in the region, it is likely to be the PKC phosphorylation site. To confirm this hypothesis, we generated an antibody against a synthetic phosphopeptide having a sequence equivalent to SNAP-25 residues 182–192, and including a phospho-serine at position 187. We purified the specific IgG from the antiserum using two affinity resins, phosphopeptide-conjugated and unphosphorylated peptide-conjugated Sepharose, as described in "MATERIALS AND METHODS." The affinity purified antibody, pAb SN25Pi, gave only a faint band at the migration position of SNAP-25 in immunoblotting of PMA-untreated PC12 cell homogenates, but gave a very intense band in PMA-treated cell homogenates (Fig. 1). In contrast, a strong band was identified in both PMA-treated and -untreated PC12 cell homogenates with BR05, a conventional monoclonal antibody to SNAP-25. The immunoreactivity of pAb SN25Pi disappeared when the antibody was preadsorbed with the antigen phosphopeptide. As shown in Fig. 1B, the intensity of the immunoreactive band decreased with increasing peptide concentrations, and was abolished in the presence of phosphopeptide concentrations greater than 0.1 μg/ml. The intensity of the band decreased by 35% in the presence of the unphosphorylated peptide at a concentration of 0.1 μg/ml, however, further decrements were not observed at higher concentrations up to 1 μg/ml. These results indicate that pAb SN25Pi specifically recognizes SNAP-25 phosphorylated at Ser^{187} in PC12 cells.

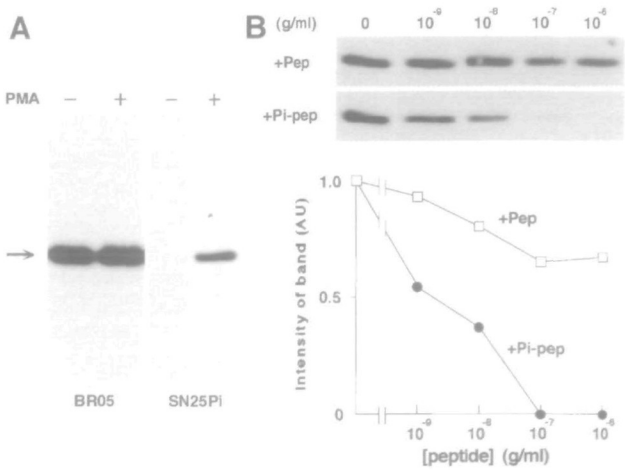

Fig. 1. **Immunoblotting of cellular homogenates of PC12 cells with or without PMA treatment using pAb SN25Pi (SN25Pi) and a monoclonal anti SNAP-25 antibody (BR05).** A, PC12 cells were incubated in the presence (+) or absence (−) of 1 μM PMA for 30 min. The cellular proteins (10 μg) were separated by SDS-PAGE and analyzed by immunoblotting with either BR05 or pAb SN25Pi. The migration position of SNAP-25 is indicated by the arrow. B, (top) PMA-treated PC12 cellular homogenates were analyzed by immunoblotting using pAb SN25Pi which had been preabsorbed with various amounts of either the antigen phosphopeptide (Pi-pep) or the unphosphorylated peptide (Pep). Immunoreactive bands were visualized using peroxidase-labeled anti mouse IgG and an enhanced chemiluminescence detection system and X-ray film. (Bottom) Intensities of the immunoreactive bands were quantified by densitometry and plotted as relative values to those obtained without preadsorption.

Imuunostaining of PC12 Cells with pAb SN25Pi—In order to know the intracellular location of the phosphorylation reaction, immunofluorescence microscopy using pAb SN25Pi and BR05 was performed in PC12 cells with or without PMA-treatment. BR05 gave strong staining in the plasma membrane and intracellular organelles, possibly the Golgi apparatus, in both PMA-treated and -untreated PC12 cells (Fig. 2, D and A). The intensity of the staining was not changed by PMA-treatment. On the other hand, only background level staining was obtained with pAb SN25Pi in PMA-untreated PC12 cells (Fig. 2B), while very strong immunoreactivity appeared, only in plasma membrane after treating the cells with PMA (Fig. 2, E and F). No significant staining was observed in intracellular organelles. These results indicate that pAb SN25Pi detects the PKC-dependent phosphorylation of SNAP-25 in PC12 cells.

Time-Dependent Changes in Neurotransmitter Release from PC12 Cells and the Phosphorylation of SNAP-25 after PMA Treatment—DA and ACh release, and the phosphorylation of SNAP-25 were measured at various times after PMA-treatment (Fig. 3A). The amount of SNAP-25 phosphorylated by PKC was estimated by quantitative immunoblotting with pAb SN25Pi. DA and ACh release were stimulated by membrane depolarization, from 0.2 to 2.4% for DA, and from 0.1 to 1.5 % for ACh, over 1 min, by changing the extra cellular K^+ concentration from 5 to 35 mM. High-K^+-dependent release of both DA and ACh increased in a time-dependent manner after treating the cells with PMA, and reached a maximum at around 3 min after treatment. The basal release of both DA and ACh in low-K^+ solution was also increased by PMA-treatment, but the increments occurred more slowly than with high-K^+-dependent release. No stimulation was observed 1 min after treatment, and release increased gradually thereafter, attaining a plateau 10 min after treatment. There were no significant differences in the time courses of potentiation between DA and ACh release. No significant phosphorylation of SNAP-25 was observed at 3 min after PMA-treatment, although it then increased gradually up to 20 min. These results indicate that PMA-dependent potentiation of high-K^+-dependent neurotransmitter release occurs more quickly than the phosphorylation of SNAP-25 by PKC.

Potentiation of Neurotransmitter Release and the Phosphorylation of SNAP-25 at Various Concentrations of PMA—Neurotransmitter release and the phosphorylation of SNAP-25 were examined at various PMA concentrations (Fig. 3B). Potentiation of high-K^+-dependent DA and ACh release occurred at relatively low concentrations of PMA, appearing above 10 nM PMA and reaching a maximum above 100 nM. On the other hand, no potentiation of basal release in low-K^+ solution was observed at 10 nM PMA, but occurred at higher PMA concentrations. Only a very low level of SNAP-25 phosphorylation was detected at 10 nM PMA, which then increased abruptly between 30 and 100 nM. There were essentially no differences in the PMA concentration–dependencies of DA and ACh release. These results indicate that the potentiation of high-K^+-dependent neurotransmitter release from PC12 cells occurs at lower PMA concentrations than those required for the phosphorylation of SNAP-25.

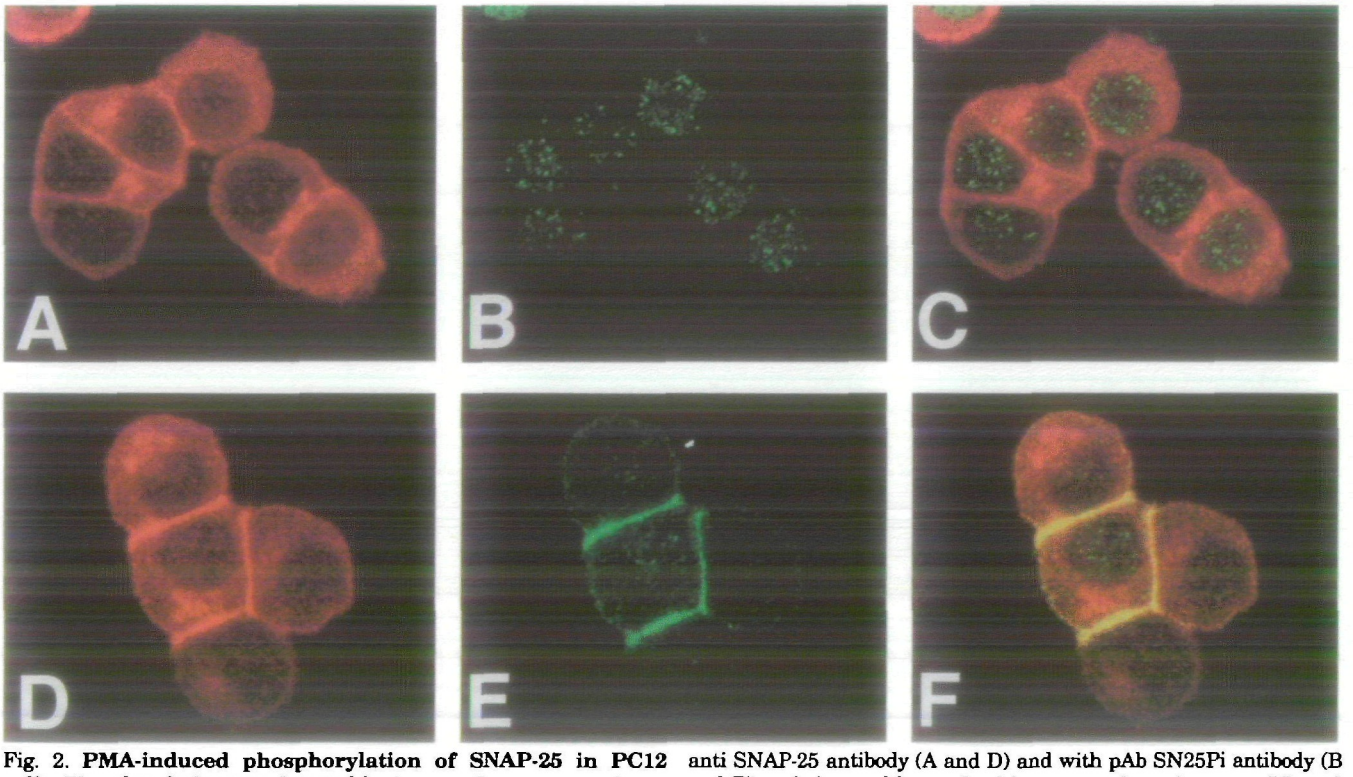

Fig. 2. **PMA-induced phosphorylation of SNAP-25 in PC12 cells.** Phosphorylation was detected by immunofluorescence microscopy using pAb SN25Pi. PC12 cells treated with (D, E, and F) or without (A, B, and C) 1 μM PMA were double-stained with a monoclonal anti SNAP-25 antibody (A and D) and with pAb SN25Pi antibody (B and E), and observed by confocal laser scanning microscopy. Merged images (C and F) show colocalization of the immunoreactivities in yellow. Bar, 20 μm.

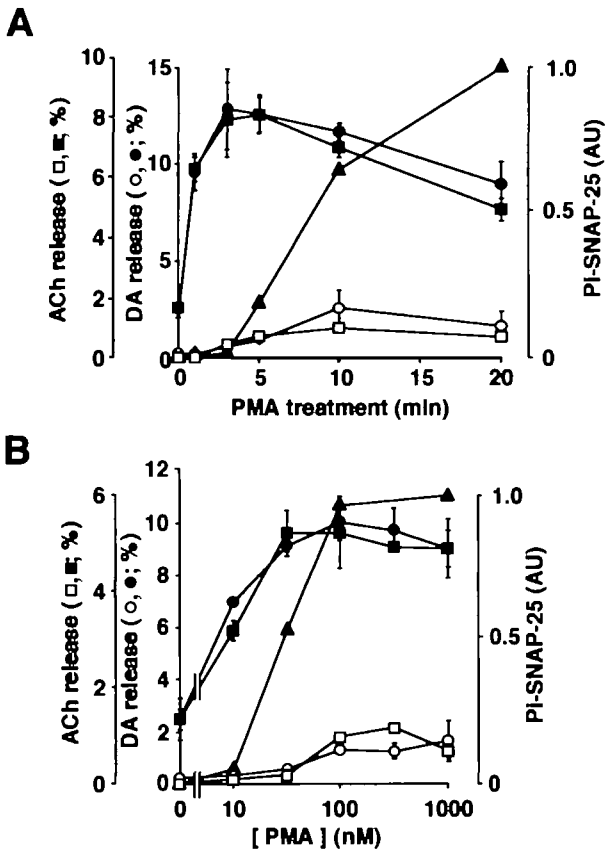

Fig. 3. **PMA-dependent stimulation of DA and ACh release from PC12 cells and phosphorylation of SNAP-25.** A, PC12 cells were preincubated with 0.1 μM PMA for the indicated periods, and challenged with either low-K^+ (○, □) or high-K^+ (●, ■) solutions for 1 min. DA (○, ●) and ACh (□, ■) released are expressed as percentages of the total cellular content. In parallel experiments, cellular homogenates prepared from PC12 cells treated with PMA for the indicated periods were analyzed by quantitative immunoblotting using pAb SN25Pi antibody. Phosphorylation (▲) is indicated relative to that obtained at 20 min incubation. B, PC12 cells were preincubated with various concentrations of PMA for 15 min, and challenged with either low-K^+ (○, □) or high-K^+ (●, ■) solutions for 1 min. DA (○, ●) and ACh (□, ■) released are expressed as percentages of the total cellular content. In parallel experiments, cellular homogenates prepared from PC12 cells treated with the indicated concentrations of PMA were analyzed by quantitative immunoblotting using pAb SN25Pi antibody. Phosphorylattion (▲) is indicated relative to that obtained in the presence of 1 μM PMA. Results are means ± SE (n = 3).

Loss of the Potentiating Effect of Phorbol Ester on Neurotransmitter Release after Wash Out—In order to study further the correlation between the potentiation of neurotransmitter release and the phosphorylation of SNAP-25 induced by PMA, time-dependent changes in the high-K^+-dependent potentiation of DA release and SNAP-25 phosphorylation were examined after washing out the phorbol ester. PDA, a less hydrophobic phorbol ester, was used in these experiments. PDA treatment induced the potentiation of high-K^+-dependent DA release and the phosphorylation of SNAP-25 as in the case of PMA (Fig. 4, A and B). At 20 min after PDA treatment, when the potentiation of DA release and the phosphorylation of SNAP-25 reached a plateau, the PDA was washed out by rinsing the cells with fresh low-K^+ solution. The phosphorylation level of SNAP-25 decreased slightly after the washing, however, it remained stable in the presence of okadaic acid, a potent phosphatase 1 and 2A inhibitor (Fig. 4B). An inactive analogue of okadaic acid, 1-norokdaone, had no effect. Interestingly, the PDA-dependent potentiation of high-K^+-induced DA release disappeared almost completely within 1 min after washing. These results clearly show that the phosphorylation of SNAP-25 at Ser^{187} is not involved in the PMA-dependent potentiation of neurotransmitter release from PC12 cells.

Effect of Staurosporine on PMA-Dependent Potentiation—PKC activated by PDA phosphorylates not only SNAP-25 but also other endogenous protein substrates, and the phosphorylation of these proteins probably persists after the PDA is washed out in the presence of okadaic acid. These results suggest the possibility that phosphorylation is not involved in the PMA-dependent enhancement of neurotransmitter release from PC12 cells. To investigate this hypothesis, the effect of a broad spectrum protein kinase inhibitor, staurosporine (*21*), on the PMA-induced potentiation of high-K^+-dependent neurotransmitter release from PC12 cells was examined. As shown in Fig. 5, 1 μM staurosporine abolished the PMA-induced phosphorylation of SNAP-25, indicating that the activity of PKC is completely inhibited by staurosporine at this concentration. Both the basal release in low-K^+ solution and the high-K^+-evoked release of DA were stimulated in the presence of PMA. The activation of basal release was suppressed in the presence of 1 μM staurosporine (Fig. 5). The basal release was also suppressed by 60% in the presense of bisindolylmaleimide (3 μM) or Ro-31-8425 (1 mM), other PKC inhibitors. In contrast, potentiation of the high-K^+-dependent release of DA was only partially inhibited by staurosporine. Figure 6 shows the concentration dependency of the staurosporine inhibition of high-K^+-dependent DA release and SNAP-25 phosphorylation. Staurosporine diminished the PMA-induced phosphorylation of SNAP-25 in a dose-dependent manner, and complete suppression was achieved at 1 μM. The PMA-induced potentiation of high-K^+-dependent DA release was also partially suppressed, and 20% inhibition was observed at 100 nM; however, no further decrease was observed even at staurosporine concentrations up to 1 μM.

DISCUSSION

In the present study, we generated an anti phosphopeptide antibody, pAb SN25Pi, and showed that Ser^{187} is the PKC phosphorylation site of SNAP-25 in PC12 cells. From quantitative analysis using pAb SN25Pi, we also demonstrated that phorbol esters potentiate neurotransmitter release from PC12 cells by two different mechanisms, one phosphorylation-dependent and the other phosphorylation-independent.

In a previous study, we ascertained that among SNARE proteins, only SNAP-25 is phosphorylated by PKC after treating PC12 cells with PMA (*14*). The phosphorylation occurred very specifically and only Ser^{187}, located between the cleavage site for BoNT/A and BoNT/E, was phosphorylated by PMA treatment. Our current data show that pAb SN25Pi appears only as a faint band in immunoblots of control PC12 cells, but gives a very intense band in blots

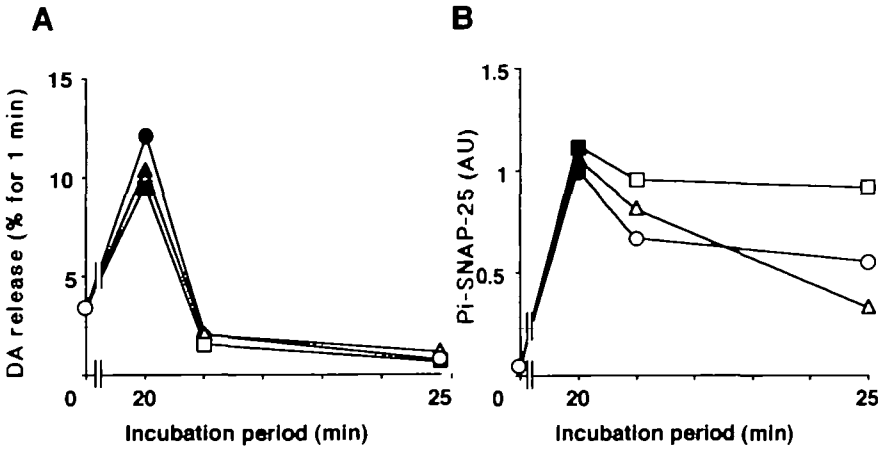

Fig. 4. **Loss of activation of neurotransmitter release after washing out the water-soluble phorbol ester, PDA.** PC12 cells were incubated with 100 nM PDA (●), 100 nM PDA and 0.5 μM okadaic acid (■), or 100 nM PDA and 0.5 μM 1-norokadaone (▲) for 20 min. Following maximum activation, the cells were incubated with low-K^+ solution without PDA (○), low-K^+ solution with okadaic acid (□), or low-K^+ solution with 1-norokdaone (△). A, high-K^+–dependent DA release over 1 min. B, the phosphorylation of SNAP-25 is expressed relative to that in the presence of PDA after 20 min incubation. Typical results of two experiments are shown.

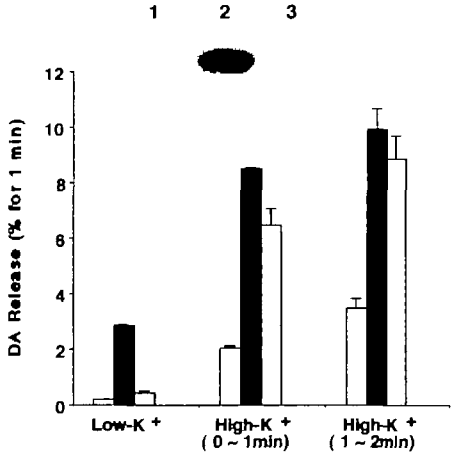

Fig. 5. **Effect of staurosporine on the PMA-induced potentiation of basal and high-K^+–evoked DA release and phosphorylation of SNAP-25.** PC12 cells were incubated in low-K^+ solution at 37°C for 30 min in the presence or absence of 1 μM staurosporine. The staurosporine-treated cells were further incubated in the presence or absence of 1 μM PMA for 20 min at 37°C. The staurosporine-untreated cells were also incubated for 20 min in the absence of either staurosporine or PMA. After the incubation, some cells were homogenized in SDS-sample buffer for immunoblotting, and others were used for DA release assay as described in "EXPERIMENTAL PROCEDURES." The amounts of DA released in the low-K^+ and high-K^+ solution over 1 min are represented as percentages of the total cellular content. Open bars, without PMA; filled bars, with PMA; shaded bars, with PMA and staurosporine. Inset, immunoblotting with pAb SN25Pi. Lane 1, without PMA; lane 2, with PMA; lane 3, with PMA and staurosporine.

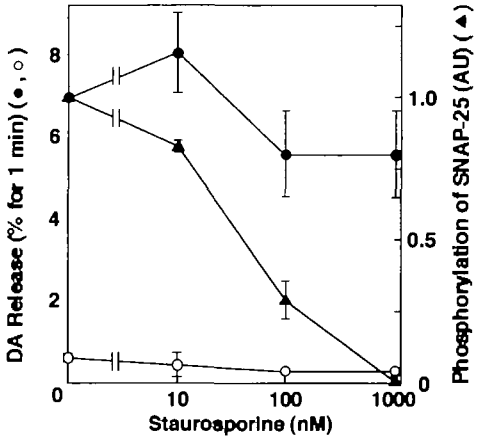

Fig. 6. **Staurosporine dose-effect curves for the inhibition of PMA-induced potentiation of DA release and phosphorylation of SNAP-25.** PC12 cells were incubated at 37°C for 30 min in the presence of various concentrations of staurosporine in low-K^+ solution. At the end of the incubation, 1 μM PMA was added and the cells were incubated for a further 20 min. After the incubation, some cells were homogenized in SDS-sample buffer for immunoblotting, and others were used for DA release assay as described in "EXPERIMENTAL PROCEDURES." The amount of DA released in the low-K^+ (○) and high-K^+ (●) solutions over 1min are represented as percentages of the total cellular content. Phosphorylation (▲) is presented relative to that in the absence of staurosporine. Results are means ± SE (n = 3).

from PMA-treated cells. The immunoreactive band was eliminated by preabsorbing the antibody with the antigen phosphopeptide, but not with the unphosphorylated peptide. The band disappeared when cells were treated with staurosporine, an inhibitor of a broad range of protein kinases, including PKC. From these results, we conclude that PKC phosphorylates Ser^{187} in PC12 cells and that pAb SN25Pi specifically recognizes SNAP-25 phosphorylated by PKC.

Phorbol ester treatment induced the potentiation of both DA and ACh release from PC12 cells. DA and ACh are stored in distinct secretory vesicles in PC12 cells, the former in large dense-core vesicles and the latter in small synaptic vesicles (*22*). The time and concentration dependencies of the effects of PMA on release were essentially the same for DA and ACh. These results indicate that exocytosis of these distinct secretory vesicles is regulated in the same manner by PMA treatment.

Quantitative analysis using pAb SN25Pi generated in the present study revealed no correlation between the enhancement of depolarization-dependent neurotransmitter release and the PKC-dependent phosphorylation of SNAP-25. The stimulation of high-K^+-dependent DA and ACh release was induced by less than 10 nM PMA, whereas the phosphorylation of SNAP-25 appeared only at PMA consentrations above 10 nM. PMA-dependent potentiation was induced within 1 min after PMA-treatment, but phosphorylation was not activated for up to 3 min after treatment. Furthermore, the potentiation of depolarization-dependent

neurotransmitter release induced by the hydrophylic phorbol ester PDA, disappeared very quickly after washing, despite full retention of SNAP-25 phosphorylation in the presence of okadaic acid. All these results indicate that the phosphorylation of SNAP-25 is not involved in the phorbol ester-induced enhancement of neurotransmitter release, and suggest that the phosphorylation of some other protein may be involved in the regulation. However, the phosphorylation of other proteins induced by PDA treatment should also be retained in the presence of okadaic acid. Furthermore, phorbol ester-induced potentiation was suppressed only partially by a universal protein kinase inhibitor, staurosporine. Taken together, these results demonstrate that protein phosphorylation is not involved in most of the phorbol ester-induced enhancement of depolarization-dependent neurotransmitter release from PC12 cells.

Phorbol esters activate PKC activity by the binding to the C1 domain of PKC (*10*). Several reports have now shown that phorbol esters potentiate vesicular transport in a phosphorylation-independent manner through binding to an unidentified C1 domain-containing protein (*23*, *24*). Recently, several phorbol ester binding proteins containing C1 domains have been identified (*25*, *26*), and Munc-13 is one good candidate for involvement in the phorbol ester-mediated regulation of neurotransmitter release (*26–33*). Munc-13 is expressed in PC12 cells (M. Kawakami and M. Takahashi, unpublished observation) and it is possible that Munc-13 is involved in the phorbol ester-mediated potentiation of neurotransmitter release from PC12 cells. However, additional studies are required to test this possibility.

In PC12 cells, PKC phosphorylates SNAP-25 exclusively at Ser^{187}, located near the C-terminus. The C-terminal region of SNAP-25 is likely to be important for certain steps in exocytosis, possibly by interacting with other presynaptic proteins such as snapin, since injection of the C-terminal peptide of SNAP-25 inhibits neurotransmitter release from various neuronal cells (*34–36*). The basal release of DA and ACh from PC12 cells observed in low-K^+ solution was also enhanced by phorbol ester, and the enhancement was abolished by treatment with various PKC inhibitors, indicating that PKC-dependent phosphorylation is involved. Thus, there is a possibility that the phosphorylation of SNAP-25 at Ser^{187} is involved in the regulation of basal release. In order to test this possibility, we are currently making a mutant mouse with a substitution in SNAP-25 of Ser^{187} to Ala.

Quite interestingly, it was reported that SNAP-25 is phosphorylated by PKC in an activity-dependent manner in cultured rat hippocampal slices (*37*). In addition to exocytosis, SNAP-25 has been shown to be involved in neurite extension (*38*). Recently, we found that NGF treatment induces the phosphorylation of SNAP-25 at Ser^{187}, as well as neurite extension in PC12 cells (*39*). It is also possible that the phosphorylation of SNAP-25 at Ser^{187} is important for the regulation of neurite extension. Further studies are necessary to examine the functional significance of SNAP-25 phosphorylation at Ser^{187}.

The present study suggests that a C1-domain-containing protein other than PKC is involved in a large proportion of the phorbol ester-mediated stimulation of neurotransmitter release. Recent reports have suggested that a C1-domain protein, possibly Munc-13, plays an important role in the phorbol ester–mediated potentiation of transmitter release from neurons (*27*, 32, *33*, *40*). On the other hand, PKC-dependent phosphorylation is certainly involved in many other preparations (*11*, *41*). The functional difference between these two types of regulation, both involving C1-domains, is not clear at present. However, it is likely that the modulation of presynaptic function depends on which types of regulatory protein exist in individual synapses. Recently, we showed that two different isoforms of complexin, which are not essential but have regulatory roles in neurotransmitter release, are differentially distributed among the synapses of cultured rat hippocampal neurons (*42*). It will be important to establish the correlation between the spectrum of expression of regulatory proteins and functional properties in individual synapses, and to examine how differential presynaptic protein distribution is regulated.

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