Synapsin I Is Phosphorylated at Ser\textsuperscript{603} by p21-activated Kinases (PAKs) \textit{in Vitro} and in PC12 Cells Stimulated with Bradykinin\textsuperscript{*}

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The function of synapsin I is regulated by phosphorylation of the molecule at multiple sites; among them, the Ser\textsuperscript{603} residue (site 3) is considered to be a pivotal site targeted by Ca\textsuperscript{2+}/calmodulin-dependent kinase II (CaMKII). Although phosphorylation of the Ser\textsuperscript{603} residue responds to several kinds of stimuli, it is unlikely that many or all of the stimuli activate the CaMKII-involved pathway. Among the several stimuli tested in PC12 cells, bradykinin evoked the phosphorylation of Ser\textsuperscript{603} without inducing the autophosphorylation of CaMKII, which was determined using phosphorylation site-specific antibodies against phospho-Ser\textsuperscript{603} synapsin I (pS603-Syn I-Ab) and phospho-Thr\textsuperscript{296/297}CaMKII. The bradykinin-evoked phosphorylation of Ser\textsuperscript{603} was not suppressed by the CaMKII inhibitor KN62, whereas high KCl-evoked phosphorylation was accompanied by CaMKII autophosphorylation and inhibited by KN62.

Thus, we attempted to identify Ser\textsuperscript{603} kinase(s) besides CaMKII. We consequently detected four and three fractions with Ca\textsuperscript{2+}/calmodulin-independent Ser\textsuperscript{603} kinase activity on the DEAE column chromatography of bovine brain homogenate and PC12 cell lysate, respectively, two of which were purified and identified by amino acid sequence of proteolytic fragments as p21-activated kinase (PAK) 1 and PAK3. The immunoprecipitants from bovine brain homogenate with anti-PAK1 and PAK3 antibodies incorporated\textsuperscript{32P} into synapsin I in a Cdc42/GTP\textsuperscript{S}-dependent manner, and its phosphorylation site was confirmed as Ser\textsuperscript{603} using pS603-Syn I-Ab. Additionally, recombinant GST-PAK2 could phosphorylate the Ser\textsuperscript{603} residue in the presence of Cdc42/GTP\textsuperscript{S}. Finally, we confirmed by immunocytochemical analysis that the transfection of constitutively active rat \(\alpha\)PAK (PAK1) in PC12 cells evokes the phosphorylation of Ser\textsuperscript{603} even in the resting mutant cells and enhances it in the bradykinin-stimulated cells, whereas that of dominant-negative \(\alpha\)PAK quenches the phosphorylation. These results raise the possibility that Ser\textsuperscript{603} on synapsin I is alternatively phosphorylated by PAKs, not only by CaMKII, in neuronal cells in response to some stimulants.

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Synapsin is a phosphoprotein found exclusively in neuronal presynaptic terminals and characterized as an anchoring protein between the vesicle phospholipid layer and the neuronal cytoskeleton. The cross-linking activity is regulated in a phosphorylation state-dependent manner, thereby controlling vesicle movement to active zones (1, 2). It has been reported that phosphorylation of synapsins is evoked by various stimuli such as psychological or electric stress (3, 4, 5), \(\beta\)-adrenergic agonists (6), and protein kinase C (PKC)\textsuperscript{3} activators (7). Isoproterenol and phorbol dibutyrate produce a dose-dependent increase in the phosphorylation of synapsin I at the Ser\textsuperscript{603} residue (site 3) (6, 7), which is recognized as the site specific for Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII) and as one of the most effective sites for neurotransmitter release (1, 2). However, it is unknown whether these stimuli directly or indirectly activate CaMKII through the adenylate cyclase/protein kinase A (PKA)-pathway or PKC-pathway(s). On the other hand, Hosaka et al. (8) proposed that neurotransmitter release may require phosphorylation of site 1 (Ser\textsuperscript{511}) in the N terminus of synapsins but not of Ser\textsuperscript{603}, the former being recognized as a PKA and CaMKII site, the latter as a CaMKII site (1, 9).

Recently, Jovanovic et al. (10) reported that brain-derived neurotrophic factor induced the release of neurotransmitter from rat synaptosomes coincidentally with the activation of mitogen-activated protein kinase and concomitantly with the phosphorylation of site 4/5 and site 6, but not with autophosphorylation of CaMKII. In contrast, Liu et al. (11) reported that brain-derived neurotrophic factor can promote the induction of long term potentiation and concomitant activation of CaMKII but not of mitogen-activated protein kinase in rat hippocampal slices, which probably occurred at a postsynaptic site. Furthermore, Matsubara et al. (12) reported that cyclin-dependent kinases phosphorylate the site 4 (Ser\textsuperscript{551}) and/or site 2 (Ser\textsuperscript{503}) on synapsin I \textit{in vitro} and that this phosphorylation affects the affinity of synapsin I to F-actin. Although the phosphorylation site essential for the release of neurotransmitters is still controversial, the Ser\textsuperscript{603} residue must be one of the pivotal sites for the release (1, 2). However, it is unlikely that many, or all of the stimuli that induce the phosphorylation of Ser\textsuperscript{603} activate the CaMKII pathway. Rather, it is plausible that many stimuli activate several protein kinases to phosphorylate the Ser\textsuperscript{603}
and dialyzed against 20 mM Tris-HCl (pH 7.5). Chromatography column lysed. His-tagged PAK2 was purified through a Ni column (Qiagen) in an intracellular Ca2+-dependent manner (13, 16). In some of these, BK stimulation seems to involve in small G protein-dependent pathway(s) in PC12 cells (17). Recently, it has been accepted that BK stimulates a small G protein Cdc42p21-activated kinase (PAK)-involved pathway in several cell systems (18–21).

In this study, we found that BK evokes the phosphorylation of synapsin I at Ser603 but not the autophosphorylation of CaMKII in PC12 cells and that bovine brain homogenate and PC12 cell lysate contain fractions that have Ca2+/calmodulin-independent Ser603 kinase activities.

**MATERIALS AND METHODS**

**Materials and Chemicals—**Synapsin I was purified from bovine brain as described by Schieberl et al. (22). GST-Cdc24 was prepared according to the method of Chuang et al. (23). His-tagged PAK2 was prepared by GeneStorm Expression-Ready clone of human PAK2 (In- vitroGen). In brief, COS7 cells were transfected with pcDNA3.1-PAK2 without 104/ml Ser 603-phosphorylated peptide (Fig. 1A). pS603-Syn I-Ab was obtained and characterized as described previously (24). In brief, Ribi adjuvant emulsion containing 25 μg of the phosphopeptide preparation was injected into BALB/c mice. Three days after the final booster, the spleen was removed and the spleen cells were fused with mouse P3UI myeloma cells. After screening for useful hybridomas by enzyme-linked immunosorbent assay (ELISA), the antibody (pS603-Syn I-Ab) obtained was subjected to immunoblot analysis with synapsin I phosphorylated by Calbicochem (San Diego, CA). Antisynapsin I antibody was from Seikagaku Kogyo (Tokyo, Japan). Other materials and chemicals were purchased from commercial sources.

**Preparation of Phosphorylation Site-specific Antibodies against Phosphorylated Synapsin I—**Monoclonal antibody against Ser603 (site 3)–phosphorylated synapsin I was raised in mice immunized with keyhole limpet hemocyanine (klh)-conjugated phosphopeptide GIPRQApSGQPGPC-klh, as previously described (24). In brief, Ribi adjuvant emulsion containing 25 μg of the phosphopeptide preparation was injected into BALB/c mice. Three days after the final booster, the spleen was removed and the spleen cells were fused with mouse P3UI myeloma cells. After screening for useful hybridomas by enzyme-linked immunosorbent assay (ELISA), the antibody (pS603-Syn I-Ab) obtained was subjected to immunoblot analysis with synapsin I phosphorylated by CaMKII and with the extract of PC12 cells stimulated with 50 mM KCl (Fig. 1, A and B). The immunoband was completely quenched with 10 μg/ml Ser603 phospho-peptide (Fig. 1A), and the cell extract showed almost a single immunoreactive band (Fig. 1B). Polyclonal antibodies (pS666-Syn I-Ab and pS603-Syn I-Ab) against synapsin I phosphorylated at either site 2 or site 3 were raised in rabbits immunized with klh-conjugated phosphopeptides ATRQApSISGPAPC-klh (site 2) and GIPRQApSGQPGPC-klh (site 3) and characterized as described previously (25). Other polyclonal antibodies were also raised in rabbits using phosphopeptides YLLRLpLDSNFMC-klh (site 1) for pS9-Syn I-Ab, ASPAAPspSGSSGCG-klh (site 5) for pS67-Syn I-Ab and CAARPAPspSGPQRG-klh (site 6) for pS551-Syn I-Ab and characterized as described above. The polyclonal antibody against Thr286cAMP phosphorylated CaMKII was prepared and characterized as described previously (26).

**Culture of PC12 Cells—**PC12 cells were plated and grown at a density of 9 × 105 cells/60 mm dish in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum and 10% horse serum at 37 °C for 2 days. After a 12-h serum starvation, the cells were used in the experiments.

**Transient Transfection of PC12 Cells—** We made use of a HA-tagged rat pPAK-encoded plasmid pXJ40HA from Dr. Manier, in which Lys296 is mutated to Ala in order to make a dominant-negative form and Thr295 is replaced with Glu to produce a constitutively active form (27). The transfection of both was conducted according to the method of Bokoch (28). Briefly, PC12 cells (1 × 106) were plated on polycarbonate-coated cover glass in 35-mm diameter dishes 24 h before transfection, washed with PBS, and then treated in 0.6 ml of Dulbecco’s modified Eagle’s medium containing 25 μl of Superfect transfection reagent (Qiagen) and 5 μg of the plasmid for 2 h at 37 °C. The resulting cells were washed with PBS and then further incubated in regular growth medium for 24 h at 37 °C. The medium was replaced with Dulbecco’s modified Eagle’s medium without serum for serum starvation overnight. The cells were subjected to the stimulation with BK or KCl and processed for immunocytochemistry.

**Immunofluorescent Staining—**PC12 cells were fixed in 4% paraformaldehyde containing 4% sucrose and 4 μg/μl GTA for 30 min at room temperature. After fixation, the cells were washed three times with PBS and then permeablized with 0.1% (v/v) Triton X-100 in PBS. After a wash with PBS, the cells were blocked with 5% bovine serum albumin in PBS for 1 h at room temperature and washed once with PBS. The cells were incubated with a 1:200 dilution of monoclonal anti-HA antibody (Santa Cruz Biotechnology) and 1:2000 dilution of polyclonal pS603-Syn I-Ab in PBS containing 5% bovine serum albumin overnight at 4 °C. They were washed three times with PBS for 5 min and then incubated with 7.5 μg/ml fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) and 0.75 μg/ml tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) for 1 h.

**Immunological Detection of Phosphorylation of Synapsin I at Ser603—** PC12 cells were washed with HEPES-buffered saline (HBS) which contained 150 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 0.8 mM MgSO4, 20 mM glucose, and 20 mM HEPES (pH 7.2) and incubated for 15 min at 37 °C. After preincubuation with or without KN62, the cells were stimulated by 1 μM BK or 50 mM KCl at 37 °C for a specified period. The reaction was terminated by adding 5% trichloracetic acid, and the cells were then scraped and sedimented. The trichloracetic acid cell pellet was dissolved in 200 μl of Laemmli sample buffer containing 8 M urea and a protease inhibitor mixture and then neutralized. 10 μl of the sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 5–20% polyacrylamide gel and then transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA.). After blocking with skimmed milk, the membrane was treated with the primary antibody (polycyclonal (1:500) or monoclonal (1:100) pS603-Syn I-Ab) followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody (Amersham Biosciences) using an ECL system (Amersham Biosciences).
only described (29), and the enzyme reaction was started by adding 
CaMKII to a 100-μl reaction mixture containing 20 μg of synapsin I, 0.1 
mM CaCl2, 3 μM calmodulin, and 0.1 mM [γ-32P]ATP or cold ATP for 10–30 
min at 30°C. The resulting mixture was subjected to autoradiography or
immunoblotting analysis using pS603-Syn I-Ab. Phosphorylated synapsin I 
was used as the standard. The assays for other protein kinases, PAK, 
Rho-kinase, and PAKs were performed as described previously (30).

Immunoprecipitation of PAK1 and PAK3 from Bovine Brain Homogenate 
and PC12 Cell Lysate—To prepare the immunoprecipitants of 
PAK1 and PAK3 from a bovine brain homogenate and PC12 cell lysate, 
anti-PAK1 and anti-PAK3 antibodies were prebound to protein 
G-Sepharose beads (Amersham Biosciences) by incubation for 1 h at 
room temperature and then washed in lysis buffer (25 mM Tris-HCl (pH 
7.5), 5 mM MgCl2, 1% Nonidet P-40, 0.1 mM DTT, and 0.1 mg/ml bovine 
serum albumin) and incubated for 30 min at 30°C. The reaction was 
terminated by adding 100 μl of 20% H2PO4, followed by washing with 
washing buffer (0.1% Triton X-100 in PBS). Phosphorylation of synapsin I 
was determined using monoclonal pS603-Syn I-Ab (1:100) followed by 
anti-mouse IgG antibody conjugated with HRP (1:1000).

Immunoprecipitation of PAK1 and PAK3 from Bovine Brain Homogenate 
and PC12 Cell Lysate—to prepare the immunoprecipitants of 
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7.5), 5 mM MgCl2, 1% Nonidet P-40, 0.1 mM DTT, and 0.1 mg/ml bovine 
serum albumin) and incubated for 30 min at 30°C. The reaction was 
terminated by adding 100 μl of 20% H2PO4, followed by washing with 
washing buffer (0.1% Triton X-100 in PBS). Phosphorylation of synapsin I 
was determined using monoclonal pS603-Syn I-Ab (1:100) followed by 
anti-mouse IgG antibody conjugated with HRP (1:1000).

RESULTS

Phosphorylation of Synapsin I at Ser603 (site 3) in PC12 Cells—Of the several agonists tested, five evoked the phosphorylation 
of synapsin I at the Ser603 residue as determined with pS603-Syn I-Ab (Fig. 1C). The Ser603 residue was phosphorylated rapidly (within 30 s) in response to KCl, nicotine, glutamate, and BK, whereas nerve growth factor-evoked phosphorylation required a longer treatment (over 1 h). Bradykinin-evoked phosphorylation of Ser603 was accompanied by little or no autophosphorylation of CaMKII. Because the phosphorylation of Ser603 is generally considered to be CaMKII-dependent, we further investigated the BK-evoked phosphorylation at Ser603 in PC12 cells (Fig. 2). Both high KCl (60 mM) and 1 μM BK evoked a transient phosphorylation, and the extent of the KCl-evoked phosphorylation was 2- to 2.5-fold higher. The KCl- 
evoked, but not BK-evoked phosphorylation, was suppressed by 3 μM KN62, a CaMKII inhibitor, whose IC50 was around 1.5 μM (Fig. 3). These findings led us to speculate that there is another pathway that might be activated by BK for the phosphorylation 
level at 0 min without KN62. Data are expressed as the mean ± S.D. of three experiments.

Fig. 2. Time course of synapsin I phosphorylation at Ser603 in PC12 cells stimulated with KCl and BK. A, representative immuno- 
oblot analysis of Ser603 phosphorylation, synapsin I content, and 
CaMKII autophosphorylation. PC12 cells were stimulated with 50 mM 
KCl and 1 μM BK, with or without preincubation with 3 μM KN62 for 15 
min; the reaction was then stopped by adding 5% trichloroacetic acid. 
The phosphorylation of synapsin I at Ser603 and autophosphorylation 
(Tyr520/522) of CaMKII were detected by immunoblot analysis using 
pS603-Syn I-Ab and anti-phosphoCaMKII antibody. B, time course of Ser603 phosphorylation in PC12 cells stimulated with KCl and BK, 
with or without 15 min preincubation with 3 μM KN62. The extent of 
phosphorylation is expressed as relative activity against the phosphoryla-

CA2+ -independent Phosphorylation of Synapsin I at Ser603 in a Bovine Brain Homogenate—We tried to detect and isolate the 
Ser603 kinase activity under Ca2+-free conditions in 100,000 × g supernatant fractions of a bovine brain homogenate by se-
quential column chromatography using an ELISA system consis-
ting of a pS603-Syn I-Ab and synapsin I-adsorbed 96-well plate 
(Fig. 4). The first DEAE-Toyopearl column chromatography 
yielded four major peaks of Ser603 kinase activity; eluted with 
0.09 M NaCl (peak I), 0.12 M NaCl (peak II), 0.175 M NaCl 
(peak III), and 0.2 M NaCl (peak IV), respectively (Fig. 4A). The 
peak I fractions reacted with anti-Rho-kinase antibody (data 
not shown). CaMKII was detected with anti-Calpain II antibody 
in fractions 10–22 (data not shown). Peak II and peak III

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FIG. 1. Immunoblot analysis of Ser603 phosphorylation 
and synapsin I content. A, representative immuno-
oblot analysis of Ser603 phosphorylation, synapsin I content, 
CaMKII autophosphorylation. PC12 cells were stimulated with 
50 mM KCl and 1 μM BK, with or without preincubation with 3 μM KN62 for 
15 min; the reaction was then stopped by adding 5% trichloroacetic acid. 
The phosphorylation of synapsin I at Ser603 and autophosphorylation 
(Tyr520/522) of CaMKII were detected by immunoblot analysis using 
pS603-Syn I-Ab and anti-phosphoCaMKII antibody. B, time course of Ser603 phosphorylation in PC12 cells stimulated with KCl and BK, 
with or without 15 min preincubation with 3 μM KN62. The extent of 
phosphorylation is expressed as relative activity against the phosphorylation 
level at 0 min without KN62. Data are expressed as the mean ± S.D. of three experiments.
fractions were further purified through a phenyl-Toyopearl column, a Source15Q column only for peak III, a heparin-Toyopearl column, and finally a hydroxyapatite column as described under "Materials and Methods." The elution pattern of peak II and III fractions from the hydroxyapatite column is shown in Fig. 4 (B and C) and the SDS-PAGE staining with Coomassie Brilliant Blue R-250 is shown in Fig. 4 (D and E). The final eluates consisted of proteins of molecular mass 68,000 Da (for peak II) and 65,000 Da (for peak III), termed p68 and p65, respectively. The p68 and p65 obtained by SDS-PAGE were subjected to peptide microsequencing. The trypsin-digested peptide of p68 was sequenced as six internal peptides as follows: EKERPEISLP, LLIQTSMNITKEQK, YMSFTSDKSAHVTR, and ELLQHQFLK; and that of p65 as four internal peptides: EKERPEISLPSDFEHTIHGFDA, SVYTR, DIKSDGTV, and STMVTPYWAPEVV. The amino acid sequences of p68 peptides fully matched the p21-activated protein kinase isoform PAK1 as compared with the Swiss-Prot database (for peak II) and 65,000 Da (for peak III), termed p68 and p65, respectively. The p68 and p65 obtained by SDS-PAGE were subjected to peptide microsequencing. The trypsin-digested peptide of p68 was sequenced as six internal peptides as follows: EKERPEISLPSDFEHTIHGFDA, SVYTR, DIKSDGTV, and STMVTPYWAPEVV. The amino acid sequences of p68 peptides fully matched the p21-activated protein kinase isoform PAK1 as compared with the Swiss-Prot database.

**Immunoprecipitation of PAK1 and PAK3 in Bovine Brain Homogenate**—As p68 and p65 were identified in the amino acid sequence of PAK1 and PAK3, respectively, we examined whether the immunoprecipitants from a bovine brain homogenate obtained using anti-PAK1 and PAK3 antibodies could phosphorylate synapsin I at the Ser603 residue. Ser603 kinase activity was detected with ELISA as described under "Materials and Methods." The bar indicates the CaMKII-containing fractions (10–22). Chromatograms of peaks II and III after hydroxyapatite column chromatography are shown in B and C, respectively. Coomassie Brilliant Blue (R-250) staining of the kinase peak fractions obtained at each chromatography step from peak II and peak III is shown in D and E, respectively. D, DEAE-Toyopearl; P, phenyl-Toyopearl; Q, Source 15Q; H, heparin-Toyopearl; HA, hydroxyapatite column chromatography.
positive and dominant-negative HA-tagged rat PAK (identical to PAK1). In PC12 cells transfection efficiency was low, so we thus investigated the phosphorylation in an immunocytochemical analysis with pS603-Syn I-Ab. The culture, which contained wild-type and mutant cells, has the advantage of detecting the phosphorylation in both cells in the same time and the field of microscope. The expression of constitutively active PAK evoked an immunostaining signal for synapsin I phosphorylation even in resting HA-positive cells and enhanced the immunostaining signal upon stimulation with BK and KCl (Fig. 8). The BK-evoked immunostaining signal was observed in a diffusing pattern in wild-type PC12 cells. In contrast, the expression of dominant-negative PAK dramatically reduced the immunostaining signal in HA-positive cells on BK stimulation (Fig. 9). However, the KCl-evoked immunostaining signal in HA-positive cells was not reduced by expression of the dominant negative form. The results were confirmed in three other separate experiments.

**DISCUSSION**

We demonstrated in this report that our system, i.e. the protein kinase-probing system facilitates the detection of the phosphorylation site-specific protein kinase of a target molecule in tissue and cell homogenate, and that it is particularly effective for proteins phosphorylated at multiple sites. The cardinal point of this system is to use a phosphorylation site-specific antibody and whole target protein-coated plates for immunoreactions. In the case of a target protein phosphorylated at multiple sites, the 32P incorporation system cannot easily detect any sharp peaks or fractions of protein kinase on column chromatography, probably yielding broad and undistinguishable peaks. In this study, we could detect and identify PAK1 and PAK3 as Ca2+/calmodulin-independent Ser603 kinases in a bovine brain homogenate. Fortunately, our protein kinase probing system detected these activities in the column chromatography fractions even in the absence of any other activators such as Cdc42/GTPyS, or Rho/GTPyS (Figs. 4 and 7), which may be capable of detecting the basal activities of Rho-kinase and PAKs, whereas the PAK1 and PAK3 immunopre-

![Figure 5](http://www.jbc.org/)

**Fig. 5.** Internal peptide microsequencing of p68 and p65. The amino acid sequence of six tryptic peptides from p68 and four from p65 was determined. The amino acid sequences are shown with the single-letter abbreviations. Identified amino acids are boxed. These sequences were compared with those of the bovine p21-activated protein kinase isoforms PAK1 and PAK3.

![Figure 6](http://www.jbc.org/)

**Fig. 6.** Synapsin I phosphorylation activity of immunoprecipitants from a bovine brain homogenate obtained with anti-PAK1 and PAK3 antibodies. A, synapsin I was incubated with each immunoprecipitant or CaMKII in a kinase assay mixture containing γ-[32P]ATP or cold ATP in the presence or absence of 0.1 mg/ml GST-Cdc42/GTPyS. After a 30-min incubation, the resulting mixture was subjected to autoradiography and immunoblotting analysis using pS603-Syn I-Ab. B, protein kinase activity of immunoprecipitants for other sites. Synapsin I was incubated with the immunoprecipitants in the presence of GST-Cdc42/GTPyS, and the reaction was stopped by adding SDS-PAGE buffer. Synapsin I was subjected to immunoblotting analyses using various phosphorylation site-specific antibodies. Note that phosphorylation at Ser9, Ser551, and Ser 603 was detected in the immunoprecipitants.
Synapsin I Phosphorylation by PAK

Fig. 8. Augmentation by expression of constitutively active aPAK of bradykinin-evoked phosphorylation of Ser$^{603}$ on synapsin I in PC12 cells. PC12 cells were transfected with HA-tagged constitutively active rat aPAK (T422E)-encoded plasmid as described under "Materials and Methods." The transfection efficiency of PC12 cells was low, so the culture contained wild-type and mutant cells. The resulting cells were stimulated for 30 sec with 1 μM BK or 50 mM KCl, and the reaction was terminated with 4% paraformaldehyde containing 4% sucrose and 4 mM EGTA applied for 30 min at room temperature. After permeabilization with 0.1% (v/v) Triton X-100, the cells were blocked with 5% bovine serum albumin and then processed to immunocytochemical double staining with monoclonal anti-HA antibody and fluorescein isothiocyanate-conjugated goat anti-mouse IgG and with polyclonal pS603-Syn I-Ab and tetramethylrhodamine isothiocyanate-conjugated donkey anti-rabbit IgG. Note that the immunostaining signal for Ser$^{603}$ phosphorylation is observed in the resting HA-positive cell, and that upon stimulation with BK the immunostaining signal is more intensive in HA-positive than that HA-negative cells. Arrows indicate transfected cells.

Fig. 9. Suppression by expression of dominant negative aPAK of BK-evoked phosphorylation of Ser$^{603}$ on synapsin I in PC12 cells. The dominant-negative mutant (K298A) cells were obtained with the same method as in Fig. 8. Note that BK stimulation does not evoke immunostaining signal in HA-positive cell and KCl-evoked phosphorylation is not affected by the expression of dominant-negative aPAK.

cipitants from a brain homogenate required Cdc42-GTP$\gamma$S in immunoblot analysis (Fig. 6). This might be due to pS603-Syn I-Ab possibly being highly specific and able to detect a native phosphorylated form of synapsin I (in ELISA) preferentially to the denatured form (in immunoblotting system). Our protein kinase probing system may be useful for detecting new protein kinase(s) in many samples in a short time.

In this study, we showed that the bovine brain homogenate contained four major Ser$^{603}$ kinases other than CaMKII (Fig. 4). CaMKII was detected by anti-CaMKII antibody in the fractions 10–22 in DEAE-column chromatography, and Rho-kinase was detected in peak I by anti-Rho-kinase antibody. Moreover, peak I was also identified as Rho-kinase by tryptic peptide analysis, and the Rho-kinase preparation from bovine brain incorporated $^{32}$P into the Ser$^{603}$ residue of synapsin I in vitro. 2 Although we could identify neither peak VI nor the shoulder between peak I and II (Fig. 4), we showed that bovine brain contains at least four protein kinases for synapsin I at Ser$^{603}$ (site 3): CaMKII, Rho-kinase, PAK1 and PAK3. These findings are surprising, because the Ser$^{603}$ site has been believed to be specific for CaMKII (1, 4, 5, 8). There is a growing body of evidence that PAKs can phosphorylate certain proteins possessing an appropriate amino acid sequence. King et al. (32, 33) have reported that PAK3 phosphorylates Raf-1 at Ser$^{338}$ whose flanking domain contains the amino acids sequence $^{333}X$RXXRX$^{338}$, and such positively charged arginines at 333 (−5 N-terminal-side of Ser$^{338}$, P-5) and 336 (P-2) are essential for phosphorylation. Similar findings concerning the consensus sequence for PAK-phosphorylation are reported for several kinds of proteins (34–36), proposing that PAK requires a basic amino acid sequence, such as arginine and lysine, at −2 or more N-terminal-side positions (up to −5) from the phosphorylation site. The Ser$^{603}$ flanking domain RXXS of synapsin I is compatible with the consensus motif for PAK-phosphorylation. Thus, these findings suggest that synapsin I is a good substrate for PAKs at least in the in vitro system.

Many laboratories have reported that PAK activity is regulated by various external stimuli through the activation of cell surface receptors, including G-protein-coupled receptors, growth factor receptors (37), and proinflammatory cytokine receptors (15), and that its activation requires the GTP-activated form of Cdc42/Rac1, a Rho family member (38, 39). The activation of such protein-involved pathways may promote the activation of peripheral membrane associated with filopodia and lamellipodia formation through the reorganization of F-actin-containing microfilaments and/or intermediate filaments (40, 41). The role of Cdc42/Rac1-PAKs in regulating cytoskeleton-based membrane activities has been defined extensively in muscle and non-muscle cell systems, and in part in neuronal cells (28). However, little is known about the precise molecular mechanism of Cdc42/Rac1-PAKs in neuronal functions other than actin-involved events such as synaptic vesicle trafficking or nerve-end membrane events. This may be due to the lack of identification of an effector of PAKs in neurons or brain. In this study, we demonstrated that the immunoprecipitants from bovine brain obtained with anti-PAK1 and PAK3 antibodies, and the recombinant PAK2 preparation phosphorylated synapsin I at Ser$^{603}$ in a Cdc42/GTP$\gamma$S-dependent manner (Fig. 6). Furthermore, we confirmed that an aPAK preparation (provided by Dr. Inagaki) phosphorylates the Ser$^{603}$ residue in vitro. 3 In neuronal PC12 cells, BK evoked the phosphorylation of synapsin I at Ser$^{603}$, which was more transient and resistant to KN62 treatment (Figs. 2 and 3), whereas the KCl-evoked phosphorylation was sensitive to KN62 treatment. In immunocytochemical analysis, we demonstrated that the expression of dominant negative aPAK reduced the BK-evoked phosphorylation of Ser$^{603}$ in the mutant cells, but not the KCl-evoked one (Fig. 9). However, it should be evaluated which kinase the dominant-negative aPAK suppresses preferentially, PAK1 or PAK3, in BK-stimulated cells. Moreover, the expression of constitutively active aPAK elicited the phosphorylation even in the resting mutant cells (Fig. 8). Taken together with other reports, the present findings allow us to speculate that BK evokes the activation of the Cdc42-PAK pathway and thereby the phosphorylation of Ser$^{603}$ on synapsin I. This is the first proposal that PAKs phosphorylate a physiologically significant site on synapsin I in neuronal cells.

PAKs were first detected while screening for binding targets of Rac and Cdc42 GTPase in the rat brain (39). Thereafter, PAK1 and PAK3 were detected in large amounts in certain

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2. K. Sakurada, H. Kato et al., unpublished data.

3. K. Kato, Y. Sasaki et al., submitted for publication.
portions of the cortex, whereas PAK2 seemed to be ubiquitously expressed in all tissues (28). At a subcellular level, PAKs may localize in actin-containing structures and regulate several cellular events such as the formation of filopodia and actin spikes, and the actin-containing meshwork in the cell periphery (42). It is known that microinjection of Cdc42He into Swiss 3T3 cells promotes the formation of peripheral actin microspikes and filopodia (21). Some of these events may be accompanied by the phosphorylation of actin-associated proteins (34–36), suggesting that PAKs co-localize with F-actin and facilitates the phosphorylation of synapse I in response to neuronal stimulation, because synapsin I is characterized as an actin binding protein.

It has been reported that BK promotes a transient increase of intracellular Ca2+ via the activation of the phosphatidylinositol signaling pathway and, consequently, induced the autophosphorylation of CaMKII in PC12 cells (45, 46). These results differ from our findings, but at the present time we do not have data to explain the discrepancy, except for the difference in experimental conditions. However, we have obtained similar results using another neuronal cell line; i.e. BK evoked the phosphorylation of synapsin I at Ser603 without autophosphorylation of CaMKII. Thus, further study is required to solve this issue.

In conclusion, we propose the possibility of at least two pathways for the phosphorylation of synapsin I at Ser603 in the brain or neuronal cells; one involves the CaMKII and the other Cdc42/PAK.

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Synapsin I Is Phosphorylated at Ser^603 by p21-activated Kinases (PAKs) in Vitro and in PC12 Cells Stimulated with Bradykinin

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