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Phosphorylation of 46-kDa protein of synaptic vesicle membranes is stimulated by GTP and Ca^{2+}/calmodulin

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Abbreviations: CaM, calmodulin; CaMK, calmodulin-dependent protein kinase; DTT, dithiothreitol; GDI, guanine nucleotide dissociation inhibitor; IEF, isoelectrofocusing; LP2 membrane, crude synaptic vesicle membrane; PKA, protein kinase A; PKC, protein kinase C; PICK-1, protein interacting with C kinase-1; PP1γ1, protein phosphatase 1γ1; SNAP-25, 25-kDa synaptosomal associated protein; SV, synaptic vesicle

Abstract

The release of neurotransmitter is regulated in the processes of membrane docking and membrane fusion between synaptic vesicles and presynaptic plasma membranes. Synaptic vesicles contain a diverse set of proteins that participate in these processes. Small GTP-binding proteins exist in the synaptic vesicles and are suggested to play roles for the regulation of neurotransmitter release. We have examined a possible role of GTP-binding proteins in the regulation of protein phosphorylation in the synaptic vesicles. GTPγS stimulated the phosphorylation of 46 kDa protein (p46) with pI value of 5.0-5.2, but GDPβS did not. The p46 was identified as protein interacting with C-kinase 1 (PICK-1) by MALDI-TOF mass spectroscopy analysis, and anti-PICK-1 antibody recognized the p46 spot on 2-dimensional gel electrophoresis. Rab guanine nucleotide dissociation inhibitor (RabGDI), which dissociates Rab proteins from SVs, did not affect phosphorylation of p46. Ca^{2+}/calmodulin (CaM), which causes the small GTP-binding proteins like Rab3A and RalA to dissociate from the membranes and stimulates CaM-dependnet protein kinase(s) and phosphatase, strongly stimulate the phosphorylation of p46 in the presence of cyclosporin A and cyclophylin. However, RhoGDI, which dissociates Rho proteins from membranes, reduced the phosphorylation of p46 to the extent of about 50%. These results support that p46 was PICK-1, and its phosphorylation was stimulated by GTP and Ca^{2+}/CaM directly or indirectly through GTP-binding protein(s) and Ca^{2+}/CaM effector protein(s). The phosphorylation of p46 (PICK-1) by GTP and Ca^{2+}/CaM may be important for the regulation of transporters and neurosecretion.

Keywords: calcium; calmodulin; guanosine triphosphate; phosphorylation; rho GTP-binding proteins; synaptic vesicles

Introduction

Synaptic transmission is the vital passages of information in the neuronal systems, and neurons release the neurotransmitters from its presynaptic nerve endings (Jahan and Sudhof, 1994). The synaptic vesicles (SV) contain the set of proteins, comprising synapsins, synaptotagmin, synaptobrevin/vesicle associated membrane protein (VAMP), synaptophysin, synaptophorin, secretory carrier membrane proteins (SCAMPs), neurotransmitter transporters, proton pump, synaptic vesicle protein 2 (SV2), and Rab3A (a Ras-related GTPase), which are important to the regulation of membrane docking and fusion for the neurosecretion (Buerfeind *et al.*, 1996). In addtition to Rab3A, RalA is also a Ras-related GTP-binding protein and known to be also abundant in SV, whose function had not been clearly elucidated (Bielinski *et al.*, 1993). However, it was recently reported that Ral GTPases regulate secretagogue-dependent exocytosis in neurocrine cells and assmbly of exocyst complexes (Moskalenko *et al.*, 2002). Moreover, it was recently proposed that RalA-exocyst complex interaction integrates the secretory and cytoskeletal pathways (Sugihara *et al.*, 2002). Other Ras-related GTPases have been also known very important for the regulation of cellular functions such as cell proliferation, differentiation, cytoskeleton reorganization, membrane trafficking, cell movement and gene regulations (Bar-Sagi and Hall, 2000).

Some proteins in SV are known to be phosphorylated and protein phosphorylation is an important regulation step in long-term synaptic plasticity and is also a direct and rapid modulation process in exocytosis in neurons and most other cell types (Turner *et al.*, 1999). Representatively, synapsin is phosphorylated by Ca^{2+}/calmodulin (CaM)-dependent protein kinase I (CaMKI), Ca^{2+}/CaM-dependent protein kinase II (CaMKII), mitogen activated protein kinase (MAPK), and protein kinase A (PKA) (Greengard *et al.*, 1993; Jovanovic *et al.*, 1996). The influxed Ca^{2+} is known to regulate the phosphorylation through CaM and CaMKII in many cases. 25-kDa synaptosomal associated protein (SNAP-25), syntaxin 1, and VAMP, which play an important role in the process of SNARE complex formation between SVs and plasma membranes, are also phosphorylated by several protein kinases (Sollner *et al.*, 1993a; 1993b). SNAP25, syntaxin 1 and VAMP are phosphorylated by CaMKII; VAMP and SNAP-25 by protein kinase C (PKC); syntaxin 1A by casain kinase II (CK2) *in vitro* (Bennett *et al.*, 1993; Nielander *et al.*, 1995; Hirling and Scheller, 1996; Shimazakin *et al.*, 1996). Moreover, SNAP-25 is phosphorylated by PKC in also PC12 cells, and was shown to decrease its interaction with syntaxin (Shimazakin *et al.*, 1996). Two soluble proteins, SNAP and NSF, are also phosphorylated *in vitro* by CaMKII, and synaptotagmin is phosphorylated by CK2 and CaMKII (Bennett *et al.*, 1993; Popoli, 1993). Although Munc18 binds to syntaxin 1A with high affinity, the phosphorylation of Munc18 by cyclin-dependent kinase-5 (cdk5) causes the disassembly of syntaxin1A- Munc18 complexes, facilitating an incorporation of released synyaxin1A into SNARE complex as a consequence (Fujita *et al.*, 1996; Shuang *et al.*, 1998; Fletcher *et al.*, 1999). Rabphilin-3A is known to interact with Rab3A, Ras-related GTP-binding protein, and is phosphorylated *in vitro* by CaMKII and PKA (Fykse *et al.*, 1995). Many proteins were phosphorylated and phosphorylated proteins were associated with altered functional properties.

Recently it was reported that GTP and GDP stimulate the phosphorylation of specific proteins in crude SVs (Lee *et al.*, 2001). In this study, we examined the possible role of GTP-binding proteins as a regulatory protein for the phosphorylation of SV proteins, and found that *protein interacting with protein kinase C-1* (PICK-1) was phosphorylated in the presence of GTPγS and Ca^{2+}/CaM. PICK-1 was originally isolated due to its interaction with protein kinase Cα (PKCα) (Staudinger *et al.*, 1995). PICK-1 is one member of growing postdensity-95 (PSD-95) disc-large zona occludens 1 (PDZ) family of synaptic proteins involved in the targeting and clustering of synaptic proteins, and it has interactions with many important transporters like monoamine, dopamine, noradrenaline, and serotonin transporters (Deken *et al.*, 2001).

Materials and Methods

Materials

$CaCl_2$, supra pure grade, was purchased from EM Science. GDPβS, and GTPγS were from Calbiochem. Glycine, SDS, Tween-20 and Tris/base, electrophoresis grade, were from Bio-Rad. PVDF membrane was from Millipore. Anti-Rab3A, RalA, SNAP25, synaptophysin, and protein phosphatase 1γ1 (PP1γ1) antibodies and peroxidase-conjugated anti-rabbit IgG antibody were purchased from Santa-Cruz. Aprotinin, leupeptin, pepstatin A, PMSF, dithiothreitol (DTT), bovine serum albumin (BSA), cyclosporin A, cyclophilin and other reagents were purchased from Sigma.

Preparation of LP2 membranes from rat brain

Crude synaptic vesicles (LP2 membranes) were prepared from the brain of rats as described with minor modifications (Huttner *et al.*, 1983; Fischer von Mollard *et al.*, 1991; Park *et al.*, 1997). About 6.4 g of the brain of 2 month-old rat (SD) was sliced in a 10 volume ice-cold buffer A (320 mM sucrose, 4 mM Hepes pH 7.4, 2 mM EGTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.1 mM PMSF). Sliced rat brain was homogenized with Potter-Elvejehm homogenizer, and centrifuged for 10 min at 9,200 g. The pellets were resuspended in 6.4 ml buffer A and 10 volume of ice-cold distilled water was added and homogenized with Potter-Elvejehm homogenizer. Then stock solutions of EGTA and Hepes pH 7.4 were added to final concentration of 2 mM and 7.5 mM respectively, and incubated for 30 min on ice to rupture the membranes. The lysates were centrifuged for 20 min at 25,000 *g*, 4°C, and the supernatant was centrifuged for 90 min at 176,000 *g*, 4°C. The membrane pellets enriched with synaptic vesicles were resuspended in 2 ml buffer B (300 mM glycine, pH 7.4, 5 mM Hepes, pH 7.4, 0.1 mM EGTA, 2 μg/ml aprotinin, and 1 μg/ml leupeptin), and called as LP2 membranes. Aliquots of the membrane solution were stored at -70°C before usage.

Purification of CaM

Calmodulin was purified from bovine brain (350 g tissue) as described (Gopalakrishna *et al.*, 1982; Park *et al.*, 2002). CaM fractions were pooled together and concentrated to 1.5 ml using ultrafiltration with PM10 membrane (Amicon). Glycerol was added to the final concentration of 10% (v/v) to the pool and the aliquots of CaM were stored in -70°C. Purified CaM

was revealed as a single band on SDS-PAGE analysis.

Purification of Rab guanine nucleotide dissociation inhibitor (GDI)

RabGDI was purified from bovine brain as described with a little modification (Sasaki *et al.*, 1990, Lee *et al.*, 2001). The brain was homogenized in 25 mM Tris, pH 7.5, 1 mM DTT, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 μg/ml pepstatin A (Buffer A). Tissue debris was removed by ultracentrifugation at 100,000 *g*, 4°C for 1 h. The clear supernatant was loaded on DEAE-sephacel column (5×40 cm). The column was washed with 8 liters of Buffer A and the GDI fraction was eluted with Buffer A containing 0.3 M NaCl. The eluted solution was treated with 40% ammonium sulfate and incubated on ice for 30 min. The 40% ammonium sulfate pellet was removed by centrifugation at 20,000 *g*, 4°C for 20 min and the supernatant was treated with 60% ammonium sulfate and incubated for 50 min on ice. The 60% ammonium sulfate pellet was saved by centrifugation at 20,000 *g*, 4°C for 20 min. The pellet was resuspended in Buffer B (25 mM Tris, pH 7.5, 1 mM DTT, 0.5 mM EDTA, 1 mM EGTA, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 μg/ml pepstatin A) containing 10% glycerol and dialyzed against this buffer. Then sodium cholate was added to 1% (w/v) in the solution to break Rab/RabGDI complex and pH of the solution was adjusted to be 8.0 by adding 1 M Trizma/base. One third of the solution was loaded on Mono-Q column (1×10 cm) equilibrated with Buffer B containing 1% sodium cholate and 10% glycerol. The column was washed with 200 ml of the same buffer and the elution was performed with a 180 ml Buffer B and 180 ml Buffer B containing 0.5 M NaCl, 1% sodium cholate and 10% glycerol using a linear gradient. About 11 mg of pure GDI was obtained from 450 *g* of bovine brain. Aliquots of purified RabGDI solution were stored at -70°C before use.

Purification of glutathione *S*-transferase (GST)-RhoGDI

pGEX2T-RhoGDI was obtained from Dr. J. H. Kim at Korea University. To purify the proteins, E.coli DH5α containing the plasmids was cultured with 0.1 mM isopropylthio-β-D-galactoside (IPTG). Bacteria were disrupted by sonication and GST-RhoGDI was purified with glutathione (GSH)-sepharose beads (Pharmacia).

Phosphorylation of proteins

Kinase reactions were performed in a kinase buffer with a minor modification (Pang *et al.*, 1988): LP2 membranes were preincubated in the 50 μl of the 50 mM Hepes buffer, pH 7.4, containing 0.5 μM $MgCl_2$, 1 mM DTT, 2 μg/ml aprotinine, 2 μg/ml leupeptin, and 0.2 mM GDPβS or GTPγS, and then 10 μCi [γ-^{32}P]-ATP was added for the phosphorylation in the presence of phosphatase inhibitors (10 mM β-phosphoglycerate and 0.1 mM Na_3VO_4) and 10 mM $MgCl_2$. The mixture was incubated at 30°C for 30 min and the reaction was stopped by the addition of SDS-PAGE sample buffer. The sample was analyzed on SDS-PAGE (14% or 7-15% gradient gel) and ^{32}P-labeled proteins were subsequently detected by autoradiography on the dried gel.

Two-dimensional gel electrophoresis and transfer to PVDF membrane

To prepare the sample protein for the two-dimensional (2-D) gel electrophoresis, cold acetone was added to protein solutions up to 80% and incubated on ice for 30 min. The proteins precipitated were collected by centrifugation at 12,000 *g*, 4°C for 20 min. The proteins were dried in air. For isoelectrofocusing (IEF), samples were solubilized in 9.5 M urea solution containing 2% Triton X-100, 5% β-mercaptoethanol, 1.6% ampholyte (pH 5-8), 0.4% ampholyte (pH 3.5-9.5) accordingly by the given instruction (Bio-Rad). The protein samples were separated in the IEF gels (diameter 1 mm×length 7 cm) electrophoresed at 700 V for 7 h, and transferred to 14% SDS-PAFE as the second dimensional gel electrophoresis (Park *et al.*, 2002). In some cases, the phosphorylated spots of autoradiography on 2-D gel were measured by using densitometer.

Dissociation Rab3A from LP2 membranes

LP2 membranes (25 μg protein) were incubated for 30 min at 30°C in 50 ml of the 50 mM Hepes, pH 7.4 containing 0.5 μM $MgCl_2$, 1 mM DTT, 2 μg/ml aprotinin, and 2 μg/ml leupeptin, with CaM in the presence of 0.1 mM $CaCl_2$, RabGDI, and RhoGDI. The reaction mixtures were centrifuged for 30 min at 100,000 *g* in a Beckman TLA45 rotor (Park *et al.*, 1997). The supernatants were stored on ice and the pellets were resuspended in 50 μl of the same buffer with brief sonication and vortexing. Rab3A and other released proteins of the supernatant and membrane pellets were measured by Western Blot using anti-Rab3A, -RalA, -PP1γ1, -SNAP25, and -synaptophysin antibodies.

Results

In an effort to examine whether GTP-binding proteins are involved in the protein phosphorylation in SV, LP2

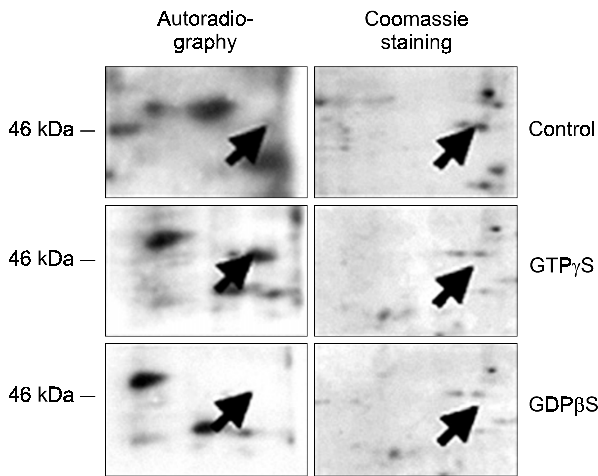

Figure 1. Guanine nucleotides regulate the phosphorylation of proteins in synaptic vesicle membrane from rat brain. 800 μg of LP2 membranes were preincubated with 0.2 mM GTPγS or 0.2 mM GDPβS at 30°C for 30 min in 675 μl of the buffer A (50 μM Hepes, pH 7.4, 0.1 mM, 0.5 μM $MgCl_2$, 1 mM DTT, 2 μg/ml aprotinine, 2 μg/ml leupeptin), and then incubated with 10 μCi [γ-^{32}P]ATP in the buffer A including 5 mM $MgCl_2$, 0.1 mM Na_3VO_4, and 10 mM β-phospho glycerate. The LP2 membranes were collected with ultracentrifuge at 100,000 *g*, 4°C for 30 min. The LP2 membranes were dissolved in isoelectrofocusing sample loading buffer (see the methods), and then isoelectrofocusing was performed with a capillary gel. Secondly, the capillary gel was run on 14% SDS-PAGE. The final gel was stained with Coomassie-blue R250, the gel was dried, and autoradiography was performed. This is a representative of at least 3 independent experiments.

membranes were incubated in the presence of GDPβS or GTPγS and then [γ-^{32}P]ATP was added to initiate phosphorylation of endogenous proteins by endogenous protein kinases in the LP2 membrane. The proteins were run on SDS-PAGE or were arrayed by the 2-D electrophoresis. It was found that GTPγS stimulated the phosphorylation of 46-kDa protein (p46) while GDPβS did not (Figure 1). Phosphorylation of p46 was not observed on SDS-PAGE, since constitutively and strongly phosphorylated 50 kDa protein disturbed the observation of p46. It was also found that phosphorylations of 70 and 100 kDa protein were stimulated by GTPγS and GDPβS, respectively, which was reported in previous paper (Lee *et al.*, 2001). Next, we tried to identify p46 by using MALDI-TOF mass spectroscopy method and searched a protein database using program NCBI and Genepept. Parts of peptides of p46 hydrolyzed by trypsin were coincided with PICK-1. From two independent analyses, PICK-1 was a common candidate protein. The predicted molecular weight of PICK-1, the original database candidate protein, was 46.5 kDa. And the molecular weight of PICK-1 was reported to be 46,552 by calculation (Staudinger *et al.*, 1995). The apparent molecular weight of PICK-1 was 46 kDa

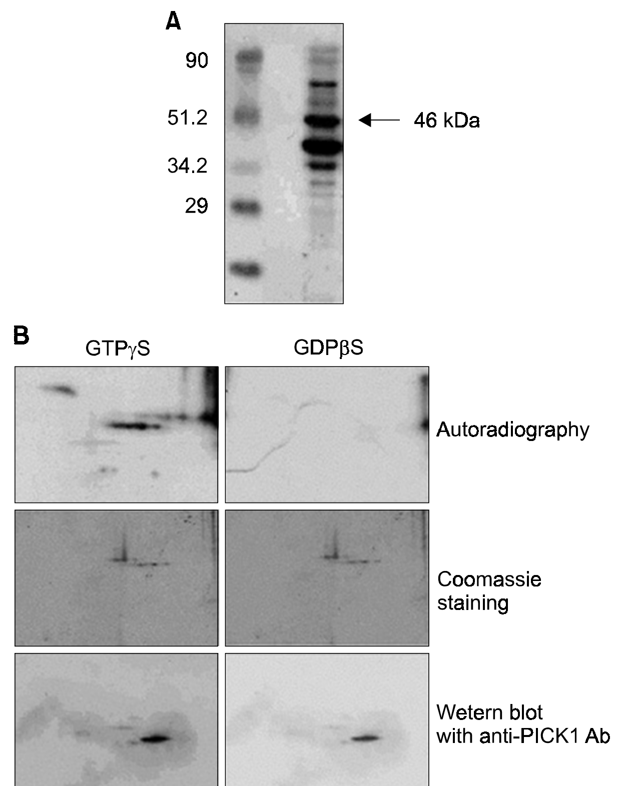

Figure 2. Western blot of 2-D gel electrophoresis membrane with anti-PICK-1 antibody. LP2 membrane (25 μg protein) was run on 14% SDS-PAGE and Western blot was performed with anti-PICK1 antibody (A). 2-D gel electrophoresis was performed using ampholytes of pH 4-6, and the proteins were transferred to PVDF membrane. Other procedures were the same as in Figure 1. The protein phosphorylation was detected by autoradiography and Western blot was executed by using anti-PICK-1 antibody (B). This is a representative of 3 independent experiments.

shown through western blot using anti-PICK-1 antibody on SDS-PAGE and 2-D gel (Figure 2A). However, apparent 40 kDa protein was also detected by anti-PICK-1 antibody, which may be thought a cleaved form of PICK-1. Phosphorylated spots of p46 were recognized by anti-PICK-1 antibody on 2-D gel electrophoresis analysis (Figure 2B), and pI values of PICK-1 were around 5.0-5.2.

To clarify whether GTP-binding proteins are involved in the regulation of the protein phosphorylation, the protein phosphorylation was performed after Rab proteins including Rab3A, a family of Ras-related small GTP-binding proteins, had been depleted from LP2 membranes by addition of RabGDI. RabGDI almost completely dissociated Rab3A from LP2 membranes and Ca^{2+}/CaM could do about 70%, whereas RhoGDI could not (Figure 3A). RabGDI was preincubated with LP2 membranes to release Rab proteins, and GTPγS was added to the LP2 membranes to load on the GTP-binding proteins. Phosphorylation

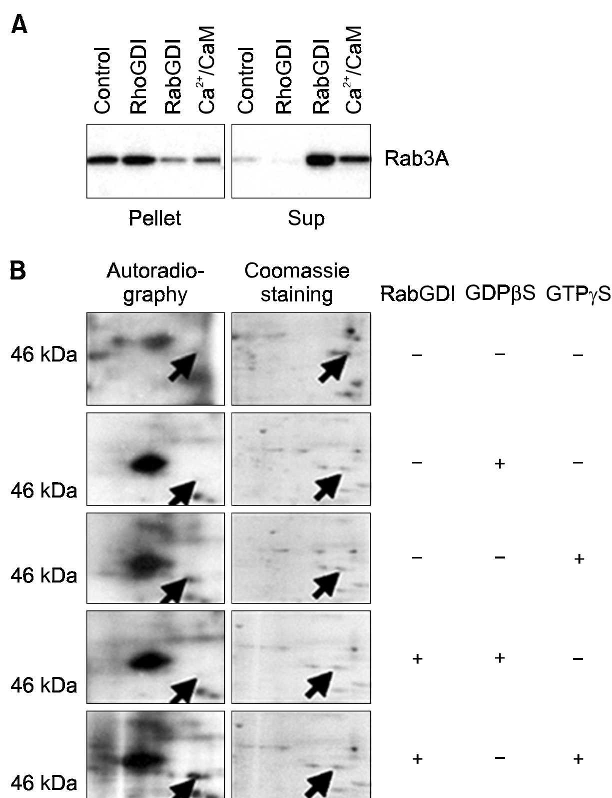

Figure 3. Dissociation of Rab3A from LP2 membranes by GDI and phosphorylation of p46 in the LP2 membranes. 25 μg of LP2 of rat brain was preincubated with 5 μg RhoGDI, 10 μg RabGDI, and 1 mM $CaCl_2$/50 μM CaM at 30°C for 30 min in 50 μl buffer A. The LP2 membrane pellet was collected with ultracentrifuge at 100,000 *g*, 4°C for 30 min, and was resuspended in 50 μl of the buffer A. The samples were run on 14% SDS-PAGE. Rab3A was detected with Western blot using anti-Rab3A antibody (A). 800 μg of LP2 of rat brain was pre-incubated with 10 μg RabGDI at 30°C for 30 min in 50 μl buffer A. The LP2 membranes were pelleted by ultracentrifuge, resuspended and incubated with 10 μCi [γ-^{32}P]ATP in the presence or absence of 0.2 mM GDPβS and GTPγS at 30°C for 30 min in 50 μl buffer A. The reacted LP2 membranes were analyzed on 2-D gel electrophoresis and authoradiography was performed with dried gel (B). This is a representative of 3 independent experiments.

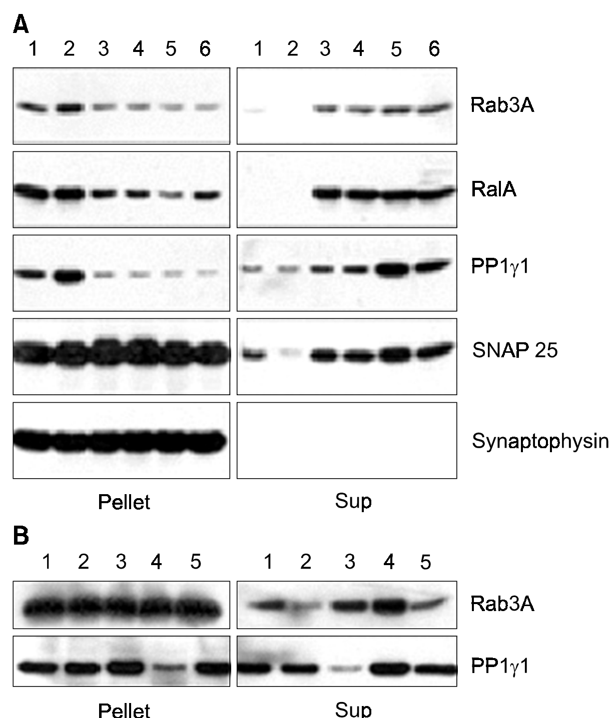

Figure 4. Ca^{2+}/CaM dissociated some proteins from LP2 membranes and stimulated the phosphorylation of 46-kDa protein in synaptic vesicle membrane of rat brain. 25 μg of LP2 of rat brain was pre-incubated with none (lane 1), 1 mM $CaCl_2$ alone (lane 2), 1 mM $CaCl_2$/48 μM CaM (lane 3), 1 mM $CaCl_2$/96 μM CaM (lane 4), 1 mM $CaCl_2$/144 μM CaM (lane 5), and 1 mM $CaCl_2$/180 μM CaM (lane 6) at 30°C for 30 min in 50 μl buffer A. The LP2 membranes were collected with ultracentrifuge at 100,000 *g*, 4°C for 30 min. The LP2 membrane pellets and supernatants were were analyzed with Western blot using anti-Rab3A, -RalA, -protein phosphatase1γ1, -SNAP25, and -synaptophysin antibodies (A). LP2 membranes were incubated with none (lane 1), 10 mM EGTA (lane 2), 1 mM $CaCl_2$ (lane 3), 1 mM $CaCl_2$/48 μM CaM (lane 4 and 5) at 30°C for 30 min in 50 μl buffer A. In addtion, the reacted solutions were incubated with none (lane 1-4), and 10 mM EGTA (lane 5) at 30°C for 30 min in 50 μl buffer A. Then the mixtures were separated to supernatant and membrane pellets by ultracentrifuge at 100,000 *g*, 4°C for 30 min. Rab3A and PP1γ1 were dectected by Western blot (B). This is a representative of 3 independent experiments.

of p46 was still active when RabGDI was added in the presence of GTPγS, while p46 was not phosphorylated in the presence of GDPβS (Figure 3B), suggesting that Rab proteins be not involved in the phosphorylation of p46.

RalA is a Ras-related GTP-binding protein and known to be also abundant in SV (Bielinski *et al.*, 1993). Thus, to test a possibility of involvement of RalA in the phosphorylation of p46, LP2 membranes was preincubated with Ca^{2+}/CaM which was known to remove RalA as well as Rab3A from membranes (Park *et al.*, 1999). Ca^{2+}/CaM dissociated many protein from LP2 membranes including Rab3A, RalA, PP1γ1 and small amounts of SNAP25, whereas synaptophysin which is membraneous protein possessing 4 transmembrane domains, could not dissociate from LP2 membranes (Figure 4A). Rab3A and PP1γ1 were returned back to the LP2 membranes when CaM became inactive by removing Ca^{2+} through chelating with excess EGTA (Figure 4B). However, p46 was still actively phosphorylated in the presence of GTPγS when Ca^{2+}/CaM was pretreated to the LP2 membranes (Figure 5). This indicates that neither RalA nor Rab proteins is involved in the stimulation of p46 phosphorylation. On the other hand, Ca^{2+}/CaM itself also induced the phosphorylation of p46 and others including 60- and 120-kDa proteins (Figure 5A, panel 3). This suggests that Ca^{2+}/CaM-dependent

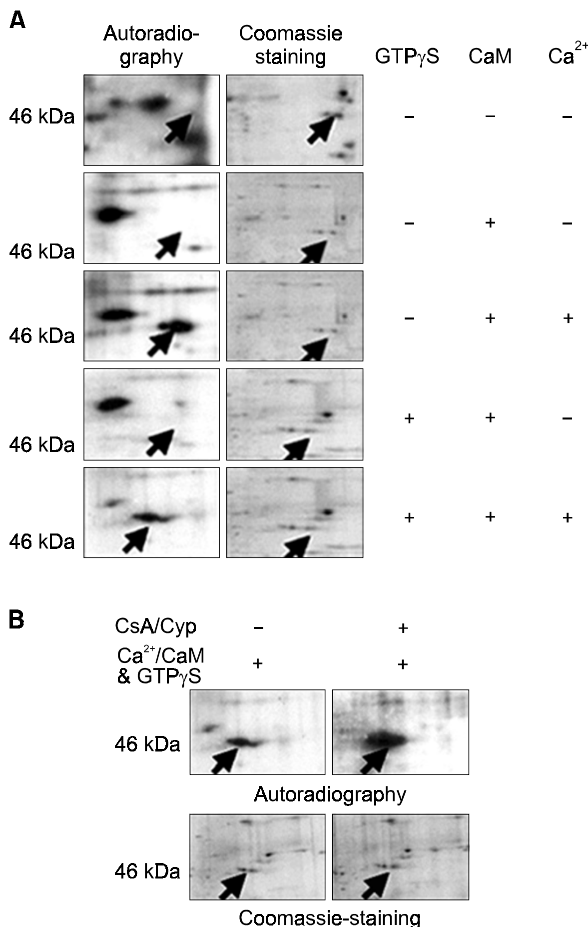

Figure 5. Ca^{2+}/CaM stimulates the phosphorylation of proteins in LP2 membranes. LP2 membranes (800 μg proteins) were incubated with none (first pannel), 48 μM CaM (second pannel), 1 mM $CaCl_2$/48 μM CaM (third pannel), 48 μM CaM and 0.2 mM GTPγS (fourth pannel), 1 mM $CaCl_2$/48 μM CaM and 0.2 mM GTPγS (last pannel) at 30°C for 30 min. And then 10 μCi [γ-^{32}P] ATP was added to initiate the phosphorylation of p46 protein by endogenous protein kinases in LP2 membranes. The reacted samples were arrayed on 2-D gel electrophoresis and then autoradiography was performed (A). 800 μg of LP2 was incubated with 1 μM cyclosporin A (CsA) and 1 μM cyclophilin (Cyp) in the presence of 1 mM $CaCl_2$/48 μM CaM and 0.2 mM GTPγS at 30°C for 30 min, and then incubated with 10 μCi [γ-^{32}P]ATP in the buffer A including 5 mM $MgCl_2$ at 30°C for 30 min. The samples were run on 14% SDS-PAGE. The gel was dried, and stained with Coomassie-blue R250. And the autoradiography was performed (B). This is a representative of 3 independent experiments.

phosphorylation of p46 is irrespective of GTP-dependent phosphorylation. CaM in the absence of Ca^{2+} could not stimulate the phosphorylation of p46 even in the presence of GTPγS (Figure 5A, panel 4). When Ca^{2+}/CaM-dependent protein phosphatase inhibitors, cyclosporin A and cyclophylin were pretreated to the LP2 membranes, these inhibitors enhanced much more phosphorylation of p46 by Ca^{2+}/CaM and GTPγS (Figure 5B).

It is known that Rho proteins could be removed from membranes by RhoGDI. To check the activity of RhoGDI to dissociate Rho proteins, RhoGDI was incubated with LP2 membranes, and RhoGDI was found to remove about 50% RhoA from LP2 membranes (Figure 6A). In the case of Cdc42, Cdc42 was much less dissociated from LP2 membranes. It was likely that pretty much portion of Rho GTPase were still present in the LP2 membrane even after treating RhoGDI to the LP2 membranes. It was observed that the phosphorylation of p46 of LP2 membranes was stimulated in the presence of GTPγS as in other results. However, RhoGDI reduced the phosphorylation of p46 to the extent of about 50% (Figure 6B, C).

Discussion

In this study, it was shown that GTPγS and Ca^{2+}/CaM stimulated 46 kDa protein of crude synaptic vesicles, irrespectively (Figure 1 and 5). MALDI-TOF mass spectroscopy analysis showed that p46 may be PICK-1. Thus we tried to confirm that p46 is PICK1 by 2-D gel analysis. Anti-PICK1 antibody could recognize the same spots of the phosphorylated p46 on 2-D gel (Figure 2B), and pI values of PICK-1 were around 5.0-5.2 estimated by 2-D gel electrophoresis, which was in accord with that previously reported as 5.21 (Staudinger *et al.*, 1995).

It is possible that GTP itself may directly bind to endogenous protein kinase of LP2 membranes. However, as was the likely case here, GTP-binding protein may regulate the protein phosphorylation by stimulation of protein kinase. When Rab GTPase were removed from LP2 membranes by GDI, phosphorylation of p46 still occurred, suggesting that Rab GTPases are not regulator in p46 phosphorylation.

Although Ca^{2+}/CaM dissociated GTPaseas like Rab3A and RalA from LP2 membranes, p46 was still phosphorylated, suggesting that RalA is not a regulator in p46 phosphorylation. On the contrary, Ca^{2+}/CaM stimulated p46 phosphorylation, suggesting that Ca^{2+}/CaM-dependent protein kinase(s) or Ca^{2+}/CaM effector protein(s) may be involved in the p46 phosphorylation irrespective of GTP-dependent process. Ca^{2+}/CaM has also been known to stimulate the protein phosphatase 2B (also called as calcineurin) as well as CaMK (Shibasaki *et al.*, 2002). To confirm this possibility, Ca^{2+}/CaM-dependent protein phosphatase inhibitors, cyclosporin A and cyclophylin were pretreated to the LP2 membranes. These inhibitors enhanced much more phosphorylation of p46 by Ca^{2+}/CaM and GTPγS (Figure 5B). These results imply that Ca^{2+}/CaM-dependent protein kinase and phosphatase simultaneously control the level of p46 phosphorylation. On the other hand, Ca^{2+}/CaM caused PP1γ1 as

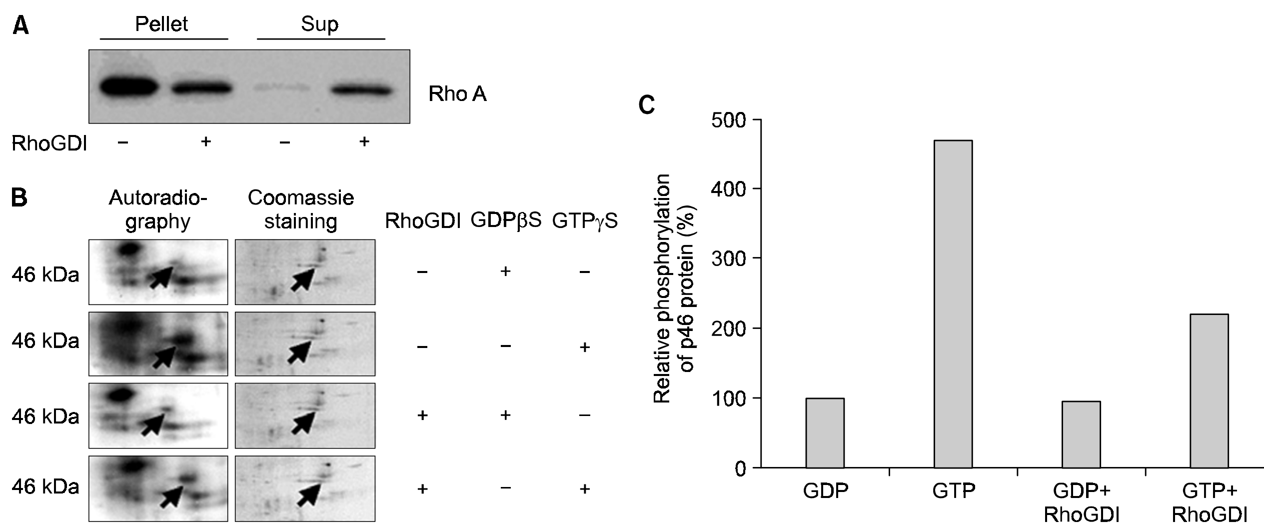

Figure 6. RhoGDI inhibited the phosphorylation of p46 protein. 800 μg of LP2 membranes were incubated with 10 μM RhoGDI at 30°C for 30 min, pelleted by ultracentrifugation, and the membrane pellets resuspended in buffer A. RhoA of a part of supernatants and membranes (25 μg protein) were determined by using Western blot (A). The LP2 membrane solutions were treated with 0.2 mM GDPβS and GTPγS, and then incubated with 10 μCi [γ-^{32}P]ATP in the buffer A including 5 mM $MgCl_2$ at 30°C for 30 min. The samples were run on 2-D gel. The gel was dried, stained with coomassie-blue R250, and the autoradiography was performed (B). This is a representative of 3 independent experiments. The p46 and 50-kDa dark spots of X-ray films (B) were determined by a densitometer, and constitutivly phosphorylated 50-kDa spot was used as each control, and the relative value of p46 comparing to 50-kDa spot in the presence of GDP was used as 100%, and other values were relatively expressed (C).

well as Rab3A and RalA to dissociate from LP2 membranes (Figure 5). It was reported that Ca^{2+}/CaM interacts with PP1γ1 forming a complex (Peters *et al.*, 1999). It is not clear whether the removal of PP1γ1 from membrane consequently leads to the increase of p46 phosphorylation. In addition, CaM in the absence of Ca^{2+}, an inactive form, could inhibit the p46 phosphorylation in the presence of GTPγS (Figure 5). One possible interpretation for this is that the inactive CaM may have an interaction with the GTP-binding protein leading to inactivation of GTP-binding protein-dependent protein kinase.

To examine whether Rho GTPases were involved in p46 phosphorylation, RhoGDI was treated with SV membranes to remove Rho GTPases. The phosphorylation of p46 was reduced about 50% although it was not completely inhibited. This suggests that Rho proteins may be involved in regulation of p46 phosphorylation. The reason that RhoGDI could not completely inhibit p46 phosphorylation might due to the presence of pretty much Rho proteins in the LP2 membranes even after treating RhoGDI. Rho family comprises many proteins like Rho (A, B, C iso forms), Cdc42, Rac (1, 2, 3 isoforms), RhoE, RhoD, RhoG, Tn10, and RhoGDI does not completely remove these proteins (Bishop and Hall, 2000): GDI shows no release activity for RhoB (Bilodeau *et al.*, 1999). However, there is still a possibility that other GTPase rather than Rho proteins may be involved in the phosphorylation of p46.

Numerous evidences support that Rho family GTPases activate many protein kinases. Rho proteins specifically activates tyrosine kinases p120^{ACK} (Manser *et al.*, 1993), p21-activated protein kinase (PAK) (Manser *et al.*, 1994), LIM-kinase which phosphorylates cofilin thereby reducing depolymerization of actin (Arber *et al.*, 1998; Yang *et al.*, 1998). In turn, PAK1 phosphorylates LIM-kinase (Edwards *et al.*, 1999). GTP-Rho activates Rho-associated serine/threonine kinase (Rho-kinase) (Matsui *et al.*, 1996), which phosphorylates myosin light chain (MLC) (Amano *et al.*, 1996a). Rho-kinase also phosphorylates myosin binding subunit (MBS) of myosin phosphatase, thereby inactivating myosin phosphatase (Kimura *et al.*, 1996). GTP-RhoA also activates serine/threonine kinase protein kinase N (PKN) (Amano *et al.*, 1996b), the substrate of which has not been clearly identified. ROKα induced the stress fiber, thereby resulting in reorganization of cytoskeleton (Leung *et al.*, 1996). However, all the substrates of these protein kinases have not been clearly elucidated albeit myosine light chain and MBS, the substrates of RhoA.

It is likely that p46 has multiple sites for phosphorylation, in that p46 showed multiple spots typically shown in Figure 1 and 5. Multiple sites of p46 may be phosphorylated by stimulation of GTPγS, Ca^{2+}/CaM through GTP-binding protein and Ca^{2+}/CaM-dependent protein kinase or Ca^{2+}/CaM effector

protein.

PICK-1 is colocalized with α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) receptors (AMPARs) at excitory synapses and is associated with AMPARs, one of glutamate receptors, in the brain (Xia *et al.*, 1999). Recent studies reveal that AMPAR trafficking is essential for synaptic plasticity that may be important for learning and memory (Luscher *et al.*, 2000). N-ethylmaleimide sensitive fusion protein (NSF) and PICK-1 binds AMPAR GluR2 subunit and are involved in trafficking of AMPARs. Recently, it was reported that GluR2, PICK-1, NSF and -/β-SNAPs form a complex in the presence of ATPγS, and the PICK1-GluR2 interactions is disrupted by NSF ATPase activity similarly to SNARE complex disassembly (Hanley *et al.*, 2002). The interaction of the presynaptic metabotropic glutamate (mGlu) receptor subtype, mGlu7, with the PDZ domain-containing protein PICK-1, is required for specific inhibition of P/Q-type Ca^{2+} channels in cultured cerebellar granule neurons (Perroy *et al.*, 2002).

Transporters can be redistributed between plasma membranes and intracellular locations in a regulated fashion. In this process, PKC is key regulator and has been known to correlate with a movement of transporters from the cell surface to intracellular locations. PICK-1 regulates transporter membrane expression, and it is substrate for PKC phosphorylation, therefore it is possible that PKC is exerting at least some of its effects on transporter expression by altering protein-protein interactions between PICK-1 and the transporter, thereby synaptic neurotransmission could be regulated (Deken *et al.*, 2001). These suggest that PICK-1 may be also involved in regulation of neuronal function and the regulation of PICK-1 phosphorylation may be essential for the neuronal function like neurosecretion and synaptic plasticity.

Conclusively, we observed that p46 protein of LP2 membranes was phosphorylated in the presence of GTPγS and Ca^{2+}/CaM, and MALDI-Tof mass spectroscopy analysis showed that p46 was PICK-1. Rho protein(s) may be involved in the phosphorylation of p46. However, it cannot be excluded that other GTPase may be involved in the phosphorylation of p46. PICK-1 phosphorylation may be essential for the neuronal function like neurosecretion and synaptic plasticity. However, it is to be elucidated that the specific protein kinase(s) and specific GTPase(s) are involved in the p46 phosphorylation. The physiological significance of the phosphorylation of p46 (PICK-1) by GTP and Ca^{2+}/CaM should be also answered in the future.

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