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Protein phosphatase 2A dephosphorylates SNAP-25 through two distinct mechanisms in mouse brain synaptosomes

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ABSTRACT

Synaptosomal-associated protein 25 (SNAP-25) plays an essential role in exocytotic neurotransmitter release as a t-SNARE protein. SNAP-25 is phosphorylated at Ser^{187} in a protein kinase C (PKC)-dependent manner, but the mechanism for dephosphorylation has yet to be clarified. We investigated SNAP-25 dephosphorylation by comparing it to growth associated protein 43 (GAP-43), another PKC-dependent presynaptic phosphoprotein, in crude mouse brain synaptosome preparations. Phosphorylation levels for both SNAP-25 and GAP-43 increased significantly after treatment with PKC activator phorbol 12, 13-dibutyrate (PDB), and ionomycin treatment induced a striking reduction in a time-dependent manner. This dephosphorylation occurred only in the presence of extracellular Ca^{2+}, indicating involvement of a Ca^{2+}-dependent phosphatase. Ca^{2+}-dependent dephosphorylation was not suppressed by calcineurin/PP2B inhibitors such as FK506 and cyclosporine A. SNAP-25 dephosphorylation, however, was suppressed by calyculin A, a non-selective inhibitor of PP1 and PP2A, and okadaic acid selective for PP2A, but not by tautomycin selective for PP1. In contrast, none of these inhibitors suppressed GAP-43 dephosphorylation. PDB-induced SNAP-25 phosphorylation was enhanced by okadaic acid in a concentration-dependent manner. These results suggest that PP2A participates in SNAP-25 dephosphorylation through Ca^{2+}-dependent and Ca^{2+}-independent mechanisms but is not involved in GAP-43 dephosphorylation.

1. Introduction

Synaptic transmission is conducted by neurotransmitters released from presynaptic terminals through exocytosis. Protein kinases have been found to activate or inhibit neurotransmitter release in various neuronal preparations (Turner et al., 1999; Takahashi and Ohnishi, 2002; Takahashi et al., 2003) and are important regulators of the synaptic plasticity underlying higher brain functions, such as learning and memory.

The synaptosomal-associated protein of 25 kDa (SNAP-25) is a t-SNARE protein essential for neurotransmitter release (Brunger, 2005; Hong, 2005; Jahn et al., 2003; Jahn & Scheller, 2006; Rizo and Rosenmund, 2008) that is phosphorylated near the C-terminus at Ser^{187} in a protein kinase C (PKC)-dependent manner (Iwasaki et al., 2000; Nagy et al., 2002; Shimazaki et al., 1996; Shu et al., 2008). We previously showed that PKC activation induced enhancement of dopamine and acetylcholine release from PC12 cells, and that Ser^{187} was phosphorylated under those conditions (Shimazaki et al., 1996; Iwasaki et al., 2000). We also found that PKC activation enhanced the recruitment of secretory vesicles containing dopamine and acetylcholine near the plasma membrane (Shoji-Kasai et al., 2002). Other studies determined that PKC activation enhanced the exocytotic release of hormones in adrenal chromaffin cells and insulin-secreting cells by increasing readily releasable and highly Ca^{2+}-sensitive vesicle pools, and that phosphorylation of SNAP-25 at Ser^{187} was essential for those effects (Gillis et al., 1996; Nagy et al., 2002; Yang et al., 2007; Shu et al., 2008). These findings suggest that SNAP-25 phosphorylation plays important roles in both nervous and endocrine systems.

SNAP-25 phosphorylation and dephosphorylation are dynamically regulated in the brain. In hippocampal slice cultures, induction of long-term potentiation for learning mechanisms accompanied a change in SNAP-25 phosphorylation (Genoud et al., 1999). SNAP-25 phosphorylation increased remarkably during early postnatal development (Kataoka et al., 2006), and mice with a single amino acid substitution of Ala for Ser^{187} had recurrent spontaneous

Abbreviations: SNAP-25, synaptosomal-associated protein 25; GAP-43, growth associated protein 43; PDB, phorbol 12,13-dibutyrate; PKC, protein kinase C.

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epileptic seizures during the same stages (Kataoka et al., 2011). In response to an intraperitoneal injection of kainate to induce seizures, SNAP-25 was rapidly phosphorylated (15–30 min) in the hippocampus, entorhinal cortex, and parietal cortex; however, the levels of phosphorylated SNAP-25 markedly decreased 60 min after injection, returning to basal levels after 3 h (Kataoka et al., 2006; Pozzi et al., 2008). Although PKC-dependent phosphorylation of SNAP-25 has been well documented, little is known about the dephosphorylation mechanism. In the present study, we examined SNAP-25 dephosphorylation in crude mouse brain synaptosome preparations (P2 membrane) and found that PP2A participates in both Ca^{2+}-dependent and Ca^{2+}-independent dephosphorylation.

2. Materials and methods

2.1. Antibodies

Anti-SNAP-25 C-terminus (Oho et al., 1995) and anti-phospho-Ser^{187}-SNAP-25 (Iwasaki et al., 2000) were prepared as previously described. Anti-phospho-Ser^{41}-growth associated protein 43 (GAP-43) was obtained by immunizing rabbits with synthetic phosphopeptide (TKIQAS[Pi]FRGHI, residues 36–46 of rat GAP-43) conjugated to keyhole limpet hemocyanin as previously described (Iwasaki et al., 2000). A cysteine residue was added to the N-terminus for conjugation. Antibodies were affinity-purified from the antiserum using an antigen peptide-conjugated Sepharose 6B column. Anti-GAP-43 antibody (mouse monoclonal, #612262) was purchased from BD Transduction Laboratories.

2.2. Reagents

The following reagents were obtained commercially: calyculin A (LC Laboratories); cyclosporine A (Santa Cruz); tautomycin (Wako Junyaku); FK506 (Cayman Chemical); and okadaic acid, ionomycin, and phorbol 12,13-dibutyrate (PDB, CalBiochem).

2.3. Animals

All experiments, unless stated otherwise, were performed in 9- to 16-week-old male C57BL/6N mice (CLEA, Japan). All procedures complied with National Institutes of Health guidelines and were approved by the Animal Experimentation and Ethics Committee of Kitasato University School of Medicine. All possible efforts were made to minimize suffering and reduce the number of mice used.

2.4. Crude mouse brain synaptosome preparations

Mice were decapitated and their forebrains quickly removed, washed once in ice-cold phosphate buffered saline, and homogenized with 12 strokes by a Teflon-glass homogenizer in ice-cold 0.32 M sucrose/5 mM HEPES (pH 7.4 with NaOH) at 900 rpm. The homogenates were centrifuged at 800 × *g* for 10 min at 4 °C, and the supernatants were further centrifuged at 10,000 × *g* for 30 min at 4 °C. The P2 membrane pellet was resuspended in a 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 D-glucose, 15 mM HEPES, and pH 7.4 with NaOH) bubbled with 100% O_2 gas.

2.5. Pharmacological signaling treatments

Crude synaptosomal membranes were preincubated at 37 °C for 10 min, followed by a 10 min incubation to phosphorylate synaptosomal proteins with either 1 μM PDB, PKC activator, or dimethylsulfoxide as a vehicle. Protein phosphatase inhibitors were added together with PDB to examine the effects. Dephosphorylation was induced for 5 min by addition of a calcium ionophore ionomycin. After incubating for various time periods at 37 °C, the membranes were solubilized in a SDS sample buffer (final concentration: 2% SDS, 3.33% glycerol, 0.002% bromophenol blue, 0.33 M Tris–HCl, and pH 7.4), boiled at 100 °C for 10 min, and stored at −80 °C.

2.6. Immunoblotting

SDS-PAGE was performed on gradient polyacrylamide gels (e-PAGEL, ATTO) in the presence of 50 mM dithiothreitol. Proteins were electrophoretically transferred onto PVDF membranes (Immobilon-P, Millipore) with a semi-dry transblotting apparatus. The membranes were blocked with 5% (w/v) non-fat milk in TBST (0.05% Tween 20, 150 mM NaCl, 25 mM Tris–HCl, and pH 7.5) and incubated with various antibodies overnight at 4 °C. After washing with TBST, the membranes were incubated for 1 h at room temperature with HRP-conjugated secondary antibodies in TBST containing 1% non-fat milk. After washing with TBST, immunoreactive bands were visualized with Super Signal chemiluminescence (Pierce) in a linear range using a luminescence image analyzer with electronically cooled CCD camera systems (LAS-4000, Fuji Photo Film Co.). Image Quant TL (Fuji Photo Film Co.) was used for image quantification of the bands.

2.7. Statistics

Data are presented as mean ± SE. Groups were compared using Tukey's honestly significant difference (HSD) post hoc tests. Statistical significance is indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3. Results

3.1. Ca^{2+}-dependent dephosphorylation of SNAP-25

We compared the dephosphorylation of SNAP-25 to GAP-43, a major PKC substrate believed to play a key role in neurite formation, regeneration, and plasticity (Benowitz and Routtenberg, 1997). Both SNAP-25 and GAP-43 are effective PKC substrates in the brain, and activation of PKC with PDB significantly increased their phosphorylation in crude mouse synaptosomes. Subsequent treatment with ionomycin, a calcium ionophore, markedly decreased the phosphorylation of both proteins (Fig. 1A-1 and B-1). Since dephosphorylation was not induced in the absence of external Ca^{2+} (Fig. 1A-2 and B-2), Ca^{2+}-dependent phosphatases were likely involved. Ionomycin-induced dephosphorylation of SNAP-25 and GAP-43 occurred in a concentration-dependent manner (Supplementary Fig. S1), with 5 μM selected for subsequent experiments.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neures.2013.01.002.

3.2. Absence of PP2B/calcineurin participation in dephosphorylation of SNAP-25 and GAP-43

PP1, PP2A, and PP2B/calcineurin are Ser/Thr phosphatases expressed in the mouse brain (Bibb and Nestler, 2006). These Phosphatases were expressed in the synaptosomal fraction as assessed by immunoblotting (Supplementary Fig. S2). Because PP2B/calcineurin is a major Ca^{2+}-dependent phosphatase in the brain, we examined the effects of PP2B/calcineurin inhibitors FK506 and cyclosporin A (Dumont, 2000; Belmeguenai and Hansel, 2005) on Ca^{2+}-dependent dephosphorylation of SNAP-25 and GAP-43. FK506 and cyclosporin A did not suppress ionomycin-induced dephosphorylation for SNAP-25 (Fig. 2A) or GAP-43 (Fig. 2B), even at concentrations up to 7.5 μM for FK506 and 49.8 μM for cyclosporin A. These results indicate that PP2B/calcineurin is not