Opposing Changes in Phosphorylation of Specific Sites in Synapsin I During Ca\textsuperscript{2+}-Dependent Glutamate Release in Isolated Nerve Terminals

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Synapsins are major neuronal phosphoproteins involved in regulation of neurotransmitter release. Synapsins are well established targets for multiple protein kinases within the nerve terminal, yet little is known about dephosphorylation processes involved in regulation of synapsin function. Here, we observed a reciprocal relationship in the phosphorylation–dephosphorylation of the established phosphorylation sites on synapsin I. We demonstrate that, in vitro, phosphorylation sites 1, 2, and 3 of synapsin I (P-site 1 phosphorylated by cAMP-dependent protein kinase; P-sites 2 and 3 phosphorylated by Ca\textsuperscript{2+}-calmodulin-dependent protein kinase II) were excellent substrates for protein phosphatase 2A, whereas P-sites 4, 5, and 6 (phosphorylated by mitogen-activated protein kinase) were efficiently dephosphorylated only by Ca\textsuperscript{2+}-calmodulin-dependent protein phosphatase 2B–calcineurin. In isolated nerve terminals, rapid changes in synapsin I phosphorylation were observed after Ca\textsuperscript{2+} entry, namely, a Ca\textsuperscript{2+}-dependent phosphorylation of P-sites 1, 2, and 3 and a Ca\textsuperscript{2+}-dependent dephosphorylation of P-sites 4, 5, and 6. Inhibition of calcineurin activity by cyclosporin A resulted in a complete block of Ca\textsuperscript{2+}-dependent dephosphorylation of P-sites 4, 5, and 6 and correlated with a prominent increase in ionomycin-evoked glutamate release. These two opposing, rapid, Ca\textsuperscript{2+}-dependent processes may play a crucial role in the modulation of synaptic vesicle trafficking within the presynaptic terminal.

Key words: 4-aminopyridine; brain-derived neurotrophic factor (BDNF); Ca\textsuperscript{2+}; calcineurin; cyclosporin A; glutamate; ionomycin; mitogen-activated protein (MAP) kinase; neurotrophins; okadaic acid; PD98059; phosphatases; synapsins; synaptosomes; neurotransmitter release

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Neurotransmitter release from nerve terminals occurs by exocytosis of synaptic vesicles mediated by protein complexes ultimately regulated in a Ca\textsuperscript{2+}-dependent manner. Synapsin I is a well characterized member of the family of neuronal-specific proteins associated with the cytoplasmic surface of small synaptic vesicles (SSVs) (De Camilli et al., 1983a,b; Huttner et al., 1983; Greengard et al., 1993) and is one of the most prominent nerve terminal phosphoproteins regulated in response to changes in intraterminal Ca\textsuperscript{2+} concentrations. It was originally identified in the brain as a substrate for multiple protein kinases (Johnson et al., 1971; Krueger et al., 1977). Protein kinase A (PKA) and Ca\textsuperscript{2+}–calmodulin-dependent protein kinase I (CaM kinase I) phosphorylate P-site-1 (Ser-9, numbering for rat synapsin I; Czernik et al., 1987) in response to activation of presynaptic second messenger cascades or Ca\textsuperscript{2+} influx, while CaM kinase II phosphorylates P-sites 2 and 3 (Ser-566 and Ser-603) in response to Ca\textsuperscript{2+} influx during nerve terminal activation (Huttner and Greengard, 1979; Sihra et al., 1989). P-sites 4 and 5 (Ser-62 and Ser-67) are phosphorylated by extracellular signal-regulated kinases (ERKs) 1 and 2, p44 and p42, of the mitogen-activated protein (MAP) kinase superfamily, whereas P-site 6 (Ser-549) is phosphorylated by MAP kinase, as well as by cyclin-dependent kinase (cdk) 1 and cdk 5, and, finally, P-site 7 (Ser-551) is phosphorylated only by cdk 5 (Jovanovic et al., 1996; Matsubara et al., 1996). MAP kinase-dependent phosphorylation of the synapsins has recently been characterized as a key step in the modulation of neurotransmitter release by brain-derived neurotrophic factor (BDNF) (Jovanovic et al., 2000). Although the role of synapsin I as a protein kinase substrate has been extensively characterized, little is known about the phosphatases involved in dephosphorylating synapsins.

In adult synapses, a variety of evidence suggests that synapsins tether a large proportion of synaptic vesicles to each other and to the actin-based cytoskeleton and thereby maintain a cluster of vesicles referred to as the “reserve pool” (Greengard et al., 1993; Hilfiker et al., 1999). A subset of vesicles, referred to as the “releasable pool”, is docked at the plasma membrane and is largely devoid of synapsin-like immunoreactivity (De Camilli et al., 1983a; Valtorta et al., 1988; Hirokawa et al., 1989; Torri-Tarelli et al., 1990, 1992; Pieribone et al., 1995). The site-specific phosphorylation of synapsins is associated with profound changes in affinity of synapsins for SSVs and for G- and F-actin (Benfenati et al., 1989). Thus, a proportion of synapsin I phosphorylated in response to Ca\textsuperscript{2+} dissociates from the vesicle membrane during sustained depolarization of synaptosomes (Sihra et al., 1989) or high-frequency stimulation in frog neuromuscular junction (Torri-Tarelli et al., 1992). Synapsin phosphorylation–dephos-
phorylation therefore likely represents a regulatory switch during SSV trafficking between these functionally distinct pools.

Here we demonstrate that Ca\(^{2+}\) influx after nerve terminal depolarization triggers a complex set of synapsin I phosphorylation–dephosphorylation reactions. Together with a rapid increase in a CaM kinase I and II–dependent phosphorylation of P-sites 1 and 2/3, a decrease in phosphorylation of P-sites 4, 5, and 6 was effected by the Ca\(^{2+}\)–calmodulin-dependent phosphatase calcineurin. Moreover, the calcineurin inhibitor cyclosporin A (CsA) prevented the dephosphorylation of P-sites 4, 5, and 6 and facilitated ionomycin-triggered release of glutamate.

**MATERIALS AND METHODS**

**Synaptosome preparation.** Synaptosomes were prepared from the cerebral cortices of two-month old male Sprague Dawley rats as described previously (Sihra, 1996). Cerebral cortices were dissected and homogenized in 0.32 M sucrose at 4°C using a Potter–Elvehjem tissue grinder with a motor-driven pestle rotated at 900 rpm. The homogenate was centrifuged at 3000 × g for 2 min at 4°C. The supernatants (S1) were centrifuged at 14,500 × g for 12 min at 4°C. The pellets (P2) were resuspended and loaded onto Percoll gradients consisting of three steps (from bottom) 23, 10, and 3% Percoll in 0.32 M sucrose additionally containing 1 μM EDTA and 250 μM HEPES. Gradients were centrifuged at 32,500 × g for 6.5 min at 4°C. Synaptosomes were harvested from the interface between the 25 and 10% Percoll layers and washed in HEPES-buffered incubation medium (HBM) (in mM): NaCl, 140; KCl, 5; NaHCO\(_3\), 5; MgCl\(_2\), 1·6H\(_2\)O, 1; NaHPO\(_4\), 1·2; glucose, 10; and HEPES, 20, pH 7.4). Washed synaptosomes were sedimented at 27,000 × g for 10 min at 4°C. The protein concentration of the resuspended pellet was determined using the Bradford assay (Bio-Rad, Hercules, CA), with bovine serum albumin as standard. Equal amounts of protein were subjected to SDS-PAGE and standard. Equal amounts of protein were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. Immunoblots were done with antibodies: P-site 1 antibody (G-257), P-site 3 antibody (RU19), P-site 4/5 antibody (PD98059 (Parke-Davis, Ann Arbor, MI), calcineurin A (Sigma, St. Louis, MO), or okadaic acid (Sigma), as noted in the legend to each figure, and each tube was placed at 37°C for 10 min. For synapsin I phosphorylation experiments, a pretreatment regimen schematically depicted below was followed (Scheme 1), in which synaptosomal pellets were resuspended in HBM containing Ca\(^{2+}\), EGTA, Co\(^{2+}\)–Cd\(^{2+}\), PDBuf (PDBuf) (Parke-Davis, Ann Arbor, MI), cyclosporin A (Sigma, St. Louis, MO), or okadaic acid (Sigma), as noted in the legend to each figure, and each tube was placed at 37°C for 10 min. The reaction 4-aminoypyridine (4-AP) was added at a reaction time of 10 min, and subsequent incubations for various times proceeded as described in the individual figure legends.

**Immunoblot analysis.** Synaptosomal samples were rapidly solubilized in 1–2% SDS (95°C), sonicated, and protein concentration was measured using BCA assay (Pierce, Rockford, IL), with bovine serum albumin as standard. Equal amounts of protein were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. Immunoblots were done with 1:500 dilutions of the following phosphorylation state-specific antibodies: P-site 1 antibody (G-257), P-site 3 antibody (RU19), P-site 4/5 antibody (G-526), and P-site 6 antibody (G-555). The specificity of these antibodies for their respective sites has been characterized previously (Czernik et al., 1987; Jovanovic et al., 1996). Total synapsin I was detected by immunoblotting with synapsin I–specific antibody (G-486; 1:500 dilution). Primary incubations were followed by incubation with \(^{125}\)I-Labeled anti-rabbit IgG (1:500 dilution; Amersham Pharmacia Biotech, Little Chalfont, UK). Blots were exposed to a PhosphorImager screen, and quantification of immunoblots was accomplished using PhosphorImager scanning and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**Dephosphorylation**

**Table 1. Site-specific synapsin I dephosphorylation by purified protein phosphatases**

<table>
<thead>
<tr>
<th>Phosphatase</th>
<th>PP1c</th>
<th>PP2Ac</th>
<th>Calcineurin</th>
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<tbody>
<tr>
<td>P-Site 1</td>
<td>1.3</td>
<td>3.8</td>
<td>7.5</td>
</tr>
<tr>
<td>P-Site 2</td>
<td>4.1</td>
<td>15.2</td>
<td>2.3</td>
</tr>
<tr>
<td>P-Site 4/5</td>
<td>1.1</td>
<td>1.5</td>
<td>8.5</td>
</tr>
<tr>
<td>Phosphorylase a</td>
<td>11.2</td>
<td>10.2</td>
<td>—</td>
</tr>
<tr>
<td>P-Thr(^{2+})-DARPP32</td>
<td>—</td>
<td>—</td>
<td>25.5</td>
</tr>
</tbody>
</table>

The activities of purified PP1c, PP2Ac, and calcineurin were measured under initial rate conditions using 1 μM \(^{32}\)P-labeled synapsin I, phospho-DARPP-32, and phospho-2 as described (Materials and Methods). Relative rates of dephosphorylation were expressed as a percentage of total \(^{32}\)P released in the presence of a phosphatase in 2.5 min. Phospho-substrates incubated in the absence of phosphatases served as control. Data represent the mean of two independent experiments, done in duplicate.

**MAP kinase assays.** Synaptosomal MAP kinase activity was assayed either by using an in-gel kinase assay as described (Jovanovic et al., 1996) or by immunoblot analysis using dual-phosphorylation state-specific, active anti-p44 and p42 MAP kinase antibody (1:10,000 dilution; Promega, Southampton, UK), followed by incubation with \(^{125}\)I-Labeled anti-rabbit IgG and visualized by PhosphorImager scanning.

In vitro **dephosphorylation.** Synapsin I was purified from bovine brain as described by Schiebler et al. (1986) and modified by Bähler and Greenberg (1987). MAP kinase, p60\(^{pp60}\)S (Sanghera et al., 1990), and the cyklin-dependent protein kinase (cdk)–cyclin A complex (Jovanovic et al., 1996) were purified from sea star oocytes and assayed as described. The catalytic subunit of PKA was purified from bovine heart as described (Kaczmar et al., 1988). The calcineurin I was purified from rat brain as described (McGuiness et al., 1985). Phosphorylation of synapsin I used the incubation conditions described for the catalytic subunit of PKA (Huttner et al., 1981), CaM kinase II (Kennedy et al., 1983; Bennett et al., 1983), MAP kinase, p44 MAP kinase, and cdk1–cyclin A (Jovanovic et al., 1996), in the presence of 150 μM ATP with trace amounts of [γ-\(^{32}\)P]ATP, to yield a final stoichiometry of 0.7, 2.4, 1.3, and 0.8 molP/mol of synapsin I, respectively. Incorporation of \(^{32}\)P was measured using PhosphorImager scanning. The phosphorylated forms of synapsin I were repurified as described (Czernik et al., 1986). Dopamine- and cAMP-regulated phosphoprotein (Mr = 32,000) (DARPP-32) phosphorylated by PKA at Thr-34 to a stoichiometry of 0.5 molP/mol of protein (Girault et al., 1989), and phosphorylase a (Cohen et al., 1988a,b) were phosphorylated and repurified as described.

In vitro **dephosphorylation.** Catalytic subunits of PP1 (PP1c, Mr = 37,000) and PP2A (PP2Ac, Mr = 38,000) were purified from rabbit skeletal muscle (Cohen et al., 1988a,b) and calcineurin (Mr = 26,000) from rat brain (Nairn et al., 1995). Purified phosphorylated were assayed in 50 mM Tris–HCl, pH 7.0, 15 mM 2-mercaptoethanol, and 1 mg/ml BSA at 30°C, as described (Desdouits et al., 1998), in the presence of 0.3% Brij-35 and 0.3 mM EGTA in the case of PP1c and PP2Ac, or 100 μM CaCl\(_2\) and 1 μM calmodulin in the case of calcineurin. Reactions were started by the addition of substrate and terminated by the addition of 200 μl of 20% (w/v) trichloroacetic acid. After the further addition of 50 μl of 10 mg/ml bovine serum albumin, samples were centrifuged for 5 min at room temperature at 17,000 × g, and the amount of \(^{32}\)P in the supernatant and the pellet was determined by measurement of Cerenkov radiation.

PP1c and PP2Ac activities were measured under initial rate conditions (the release of phosphate was linear with time and enzyme concentration and corresponded to <25% of the phosphate incorporated into the substrate), and used 1 μM \(^{32}\)P-phosphophorylase a as substrate (Ingelbretn et al., 1983). For measurements of calcineurin activity, initial rate conditions were determined using 1 μM \(^{32}\)P-phospho-DARPP-32. Under the same conditions, PP1c-, PP2Ac- and calcineurin-catalyzed dephosphorylation of different \(^{32}\)P-labeled phospho-forms of synapsin I (1 μM) was directly compared with dephosphorylation of the standard substrates (Table 1). Data represent the means of two independent experiments, each done in duplicate.

For kinetic analysis, dephosphorylation assays were started by the addition of enzyme and performed with various concentrations of phos-
Table 2. Kinetic analysis of site-specific synapsin I dephosphorylation by purified protein phosphatases

<table>
<thead>
<tr>
<th>PPIc</th>
<th>PP2Ac</th>
<th>Calcinurin</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K_m (μM)</td>
<td>k_cat (sec⁻¹)</td>
<td>k_cat/K_m (1/sec μM)</td>
</tr>
<tr>
<td>P-Site 1</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>P-Site 2,3</td>
<td>1.8</td>
<td>21</td>
</tr>
<tr>
<td>P-Site 4,5,6</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>P-Site 6</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

The activities of PPIc, PP2Ac, and calcineurin were measured under initial rate conditions for 5 min using various concentrations of [32P]-labeled phospho-synapsin I as described (see Materials and Methods). Phospho-substrates incubated in the absence of phosphatases served as control. Kinetic parameters were calculated from linear regression analysis of Lineweaver–Burk plots, each representing the means of two independent experiments, each done in duplicate. *Indicates activities too low for accurate kinetic measurements.

RESULTS

Synapsin I dephosphorylation by protein phosphatases

The physiological implications of synapsin I phosphorylation at specific sites prompted us to identify the protein phosphatase or phosphatases responsible for dephosphorylation of these sites. In vitro analyses of the dephosphorylation of synapsin I by the purified catalytic subunits of PP1 (PPIc), PP2A (PP2Ac), and calcineurin were performed with four different phospho-forms of synapsin I: synapsin I phosphorylated at P-site 1 by PKA (P-site 1-phosphosynapsin I), synapsin I phosphorylated at P-sites 2 and 3 by CaM kinase II (P-site 2,3-phosphosynapsin I), synapsin I phosphorylated at P-sites 4, 5, and 6 by MAP kinase (P-site 4,5,6-phosphosynapsin I), and synapsin I phosphorylated at P-site 6 by cdk 1 (P-site 6-phosphosynapsin I). In these experiments the initial rates of dephosphorylation of various [32P]-phospho forms of synapsin I and [32P]-phospho-DARPP-32 or [32P]-phosphorylase a used as reference substrates, were assessed by measuring the release of phosphate (Table 1). P-site 1-phosphosynapsin I was a good substrate for PP2Ac, yet a poor substrate for either PPIc or calcineurin. P-site 2,3-phosphosynapsin I was an excellent substrate for PP2Ac and a poorer substrate for either PPIc or calcineurin. P-site 4,5,6-phosphosynapsin I and P-site 6-phosphosynapsin I were most efficiently dephosphorylated by calcineurin. These initial results were extended by a more complete kinetic analyses of dephosphorylation of the four phospho-forms of synapsin I by PPIc, PP2Ac, and calcineurin (Table 2). Three kinetic parameters, K_m (the apparent affinity for substrate), k_cat (the turnover number), and k_cat/K_m (the catalytic efficiency) were determined from linear regression analysis of Lineweaver–Burk transformations of data describing the initial rates of dephosphorylation as a function of substrate concentrations. Values obtained for phospho-DARPP-32 and phosphorylase a as standards were similar to those reported previously (King et al., 1984) (data not shown). P-site 1-phosphosynapsin I and P-site 2,3-phosphosynapsin I were high-affinity substrates for PP2Ac with very high turnover numbers (k_cat) and catalytic efficiencies (k_cat/K_m). P-site 2,3-phosphosynapsin I was also a high-affinity substrate for PPIc, but with a significantly lower k_cat. In contrast, these phospho-forms of synapsin I were both poor substrates for calcineurin. P-site 4,5,6-phosphosynapsin I and P-site 6-phosphosynapsin I were efficiently dephosphorylated by calcineurin, with k_cat values and catalytic efficiencies similar to those obtained for dephosphorylation of phospho-Thr34-DARPP-32. Previous studies of P-site 1-phosphosynapsin I and P-site 2,3-phosphosynapsin I dephosphorylation by purified protein phosphatases yielded qualitatively similar results; PP2Ac was the most effective phosphatase, calcineurin was active, although less effective at these sites, and neither phospho-form was dephosphorylated significantly by PPIc or PP2A (A. C. Nairn, H. C. Hemmings Jr, and P. Greengard, unpublished observations).

The potential involvement of PP2C in dephosphorylation of various phosphorylated forms of endogenous synapsin I was investigated using synaptosomal lysates. Synaptosomes were incubated under standard conditions for 10 min and then depolarized by the addition of 1 mM 4-AP. Samples, taken before and 1 min after the addition of 4-AP, were lysed using hypotonic conditions in the presence of 0.5 μM okadaic acid and 2 mM EGTA for 10 min at 4°C to inhibit endogenous activities of PPI, PP2Ac, and calcineurin. Synaptosomal lysates were then incubated in the presence or absence of 10 mM MgCl_2 for 10 min, under experimental conditions required for PP2C activity (Desdouits et al., 1998). Immunoblotting using phosphorylation state-specific antibodies showed no apparent change in the phosphorylation state of any of the P-sites in synapsin I in the presence of 10 mM MgCl_2 (data not shown).

4-Aminopyridine-induced changes in synapsin I phosphorylation in intact synaptosomes

Site-specific changes in the phosphorylation state of synapsin I in an isolated nerve terminal preparation (synaptosomes) were
monitored by immunoblot analysis using phosphorylation state-specific antibodies for P-sites 4/5 and 6 in synapsin I. Synaptosomes were incubated in the absence or presence of 50 μM PD98059 at 37°C, in HEPES-buffered medium containing either 1 mM CaCl₂ (Ca²⁺) or 1 mM EGTA in the absence of added Ca²⁺ (EGTA). After 10 min of incubation, 4-AP (1 mM) was added for an additional 1 min. Equal amounts of total protein (60 μg) were subjected to SDS-PAGE and immunoblot analysis using phosphorylation state-specific antibodies and 125I-labeled Protein A for detection. Results are representative of three independent experiments. A, Phosphorylation state of MAP kinase-specific P-sites 4/5 in synapsin I was analyzed using immunoblotting with P-site 4/5 antibody (G-526) (1:500 dilution). B, Phosphorylation state of MAP kinase/cdk5-dependent P-site 6 in synapsin I was analyzed using P-site 6 antibody (G-555) (1:500 dilution). C, Activities of MAP kinase isoforms ERK 1 and 2 were analyzed using immunoblotting with anti-active MAP kinase antibody (1:10,000 dilution; Promega).

Figure 1. 4-Aminopyridine-evoked depolarization and Ca²⁺ influx in synaptosomes result in a prominent dephosphorylation of MAP kinase-dependent P-sites 4/5 and 6 in synapsin I. Synaptosomes were incubated in the absence or presence of 50 μM PD98059 at 37°C, in HEPES-buffered medium containing either 1 mM CaCl₂ (Ca²⁺) or 1 mM EGTA in the absence of added Ca²⁺ (EGTA). After 10 min of incubation, 4-AP (1 mM) was added for an additional 1 min. Equal amounts of total protein (60 μg) were subjected to SDS-PAGE and immunoblot analysis using phosphorylation state-specific antibodies and 125I-labeled Protein A for detection. Results are representative of three independent experiments. A, Phosphorylation state of MAP kinase-specific P-sites 4/5 in synapsin I was analyzed using immunoblotting with P-site 4/5 antibody (G-526) (1:500 dilution). B, Phosphorylation state of MAP kinase/cdk5-dependent P-site 6 in synapsin I was analyzed using P-site 6 antibody (G-555) (1:500 dilution). C, Activities of MAP kinase isoforms ERK 1 and 2 were analyzed using immunoblotting with anti-active MAP kinase antibody (1:10,000 dilution; Promega).

Figure 2. Time course of Ca²⁺-dependent dephosphorylation of synapsin I at MAP kinase-dependent P-sites 4/5 and 6 in synaptosomes. Synaptosomes were incubated for 10 min under standard conditions in the presence of 1 mM CaCl₂ (Ca²⁺) or 0.2 mM EGTA in the absence of added Ca²⁺ (EGTA). 4-AP (1 mM, arrow) was added, and samples were collected at various time points and lysed in 1% SDS. Equal amounts of total protein (60 μg) were analyzed using SDS-PAGE and immunoblotting with P-site 4/5 (G-526) or P-site 6 Ab (G-555) (1:500 dilution). Under Ca²⁺-free conditions no significant change in the level of phosphorylation of sites 4/5 or site 6 was observed. A, Time course of Ca²⁺-dependent dephosphorylation of synapsin I P-sites 4/5 occurs rapidly resulting in a decrease to 29.6 ± 2.4% (mean ± SEM; n = 4), 1 min after depolarization by 4-AP. B, Time course of Ca²⁺-dependent dephosphorylation of synapsin I P-site 6 results in a decrease to 58.6 ± 2.9% (mean ± SEM; n = 4), 1 min after depolarization by 4-AP.
creases in the levels of phosphorylation of P-site 1 (Fig. 3A) and P-site 3 (Fig. 3B) were observed. In Ca²⁺-free conditions no significant change in the level of phosphorylation of P-site 1 or P-site 3 was observed. A, Time course of Ca²⁺-dependent phosphorylation of synapsin I at P-site 1 results in an increase of 2.08 ± 0.25-fold (mean ± SEM; n = 3) in the level of phosphorylation of P-site 1, reaching a maximal increase 10 sec after depolarization by 4-AP. B, Time course of Ca²⁺-dependent phosphorylation of synapsin I at P-site 3 occurs rapidly resulting in an 11.1 ± 2.4-fold (mean ± SEM; n = 3) increase in the level of phosphorylation of P-site 3, reaching a maximal increase 1 min after depolarization by 4-AP.

which was detected under basal conditions, showed a significant Ca²⁺-dependent increase in response to depolarization (Fig. 3A, P-site 1). Under basal conditions, P-site 3 was in a dephosphorylation state but underwent a dramatic increase in phosphorylation after nerve terminal depolarization (Fig. 3B, P-site 3). Maximal increases in the levels of phosphorylation of P-site 1 (Fig. 3A, Ca²⁺) and P-site 3 (Fig. 3B, Ca²⁺) were 2.1 ± 0.3-fold (mean ± SEM; n = 3) and 11.1 ± 2.4-fold (mean ± SEM; n = 3), respectively, and occurred with similar fast kinetics. In the presence of EGTA, the increase in phosphorylation of P-site 1 (Fig. 3A) and the rapid phosphorylation of P-site 3 (Fig. 3B) were completely inhibited. Thus, presynaptic Ca²⁺ entry has reciprocal effects on the phosphorylation state of specific P-sites of synapsin I, increasing phosphorylation at P-sites 1, 2, and 3, while decreasing phosphorylation on P-sites 4, 5, and 6.

Figure 3. Ca²⁺-dependent phosphorylation of synapsin I at CaM kinase-dependent P-sites 1 and 3 in synaptosomes. Synaptosomes were incubated for 10 min under standard conditions in the presence of 1 mM CaCl₂ (Ca²⁺) or 0.2 mM EGTA in the absence of added Ca²⁺ (EGTA). 4-AP (1 mM, arrow) was added, and samples were collected at various time points and lysed in 1% SDS. Equal amounts of total protein (60 µg) were analyzed using SDS-PAGE and immunoblotting with P-site 3 antibody (G-257) (1:500 dilution). In Ca²⁺-free conditions no significant change in the level of phosphorylation of P-site 1 or P-site 3 was observed. A, Time course of Ca²⁺-dependent phosphorylation of synapsin I at P-site 1 results in an increase of 2.08 ± 0.25-fold (mean ± SEM; n = 3) in the level of phosphorylation of P-site 1, reaching a maximal increase 10 sec after depolarization by 4-AP. B, Time course of Ca²⁺-dependent phosphorylation of synapsin I at P-site 3 occurs rapidly resulting in an 11.1 ± 2.4-fold (mean ± SEM; n = 3) increase in the level of phosphorylation of P-site 3, reaching a maximal increase 1 min after depolarization by 4-AP.

Figure 4. Effect of okadaic acid on phosphorylation state of synapsin I at P-sites 1, 3, 4/5, and 6 in synaptosomes. A, Okadaic acid (OA; 0.5 µM) was added before the transition of synaptosomes to 37°C, and the incubation was performed under standard conditions (1 mM CaCl₂) for 10 min followed by the addition of 1 mM 4-AP. Samples were collected before and 1 min after the addition of 4-AP and analyzed by SDS-PAGE and immunoblotting with phosphorylation state-specific antibodies followed by 125I-Protein A for detection. Results are representative of three independent experiments. Phosphorylation of synapsin I was analyzed using immunoblotting with P-site 1, 3, 4/5, and 6 phosphorylation-state-specific antibodies (1:500 dilution). SDS-PAGE migration of the doublet of synapsin Ia and Ib (syn I) was analyzed using immunoblotting with synapsin I specific antibody (G-486) (1:500) (syn I). Hyperphosphorylation of synapsin I at all five P-sites in the presence of okadaic acid resulted in broad bands of higher apparent molecular mass. B, Synaptosomal samples were collected before or 10 min after the transition of synaptosomes to 37°C in the presence or absence of okadaic acid (0.5 µM). Activities of MAP kinase isoforms ERK 1 and 2 were analyzed by an in-gel kinase assay with myelin basic protein as a substrate incorporated within the gel and in the presence of 40 µM [γ-32P]ATP.

Modulation of synapsin I phosphorylation by protein phosphatase inhibitors in intact synaptosomes

We used synaptosomes to investigate the effect of either okadaic acid-sensitive (inhibition of PP2A and PP1), or cyclosporin A-sensitive (inhibition of calcineurin) phosphatase activities on the phosphorylation of synapsin I at specific P-sites. Okadaic acid (0.5 µM) was added before the transition of synaptosomes to 37°C, and the incubation was performed for 10 min. Samples were collected before (control) and 1 min after the addition of 4-AP. A prominent increase in the state of phosphorylation of P-sites 1 and 3 was observed with okadaic acid treatment (Fig. 4A, P-site 1 and P-site 3) likely because of a specific inhibition of PP2A, given the concentration of okadaic acid used herein (Nishi et al., 1999). However, under the same conditions, an increase in phosphorylation state of P-sites 4/5 and 6 was also observed (Fig. 4A, P-site 4/5 and P-site 6). The latter effect was likely attributable to an increase in MAP kinase activity itself, given that these sites were
very poor substrates for PP1 and PP2A in vitro. To confirm this, we compared the activity of synaptosomal MAP kinases in the absence or presence of okadaic acid using an in-gel kinase assay with myelin basic protein as substrate (Jovanovic et al., 1996). A large increase in the activities of both p44 and p42 isoforms of MAP kinases resulted in a decrease to 58.6 ± 4.8% of control (mean ± SEM; n = 4), 1 min after depolarization by 4-AP (control). This effect was completely inhibited in the presence of cyclosporin A, resulting in a small increase to 119.4 ± 4.6% of control (mean ± SEM; n = 4), 1 min after depolarization by 4-AP (CsA).

Figure 5. Ca$^{2+}$-dependent dephosphorylation of synapsin I at MAP
kinase-dependent P-sites 4/5 and 6 in synaptosomes is completely blocked
by the calcineurin inhibitor cyclosporin A (CsA). Synaptosomes were
incubated for 10 min in the presence of 0.2 mM EGTA and in the absence
or presence of cyclosporin A (1 μM) and then depolarized with 4-AP just
before the addition of 1.2 mM CaCl₂. Control samples were collected
before the addition of 4-AP. The effect of Ca$^{2+}$ entry was than followed
at 30 sec, 1 min, and 10 min using SDS-PAGE and immunoblotting with
P-site specific antibodies. A, Ca$^{2+}$-dependent dephosphorylation of P-site

4/5 resulted in a decrease to 58.6 ± 4.8% of control (mean ± SEM; n = 4), 1 min after depolarization by 4-AP (control). This effect was com-
pletely inhibited in the presence of cyclosporin A resulting in a small
increase to 119.4 ± 4.6% of control (mean ± SEM; n = 4), 1 min after
depolarization by 4-AP (CsA).

Figure 6. Inhibition of synaptosomal calcineurin activity by cyclosporin
A correlates functionally with an increase in ionomycin-triggered glutam-
ate release. Glutamate release was triggered from rat synaptosomes (0.3
mg/1.5 ml) incubated in the presence of CoCl₂ (10 μM) and CdCl₂ (10
μM) and in the absence or presence of cyclosporin A (1 μM) for 10 min
and assayed by on-line fluorometry, as described in Materials and Meth-
ods. CaCl₂ (1 mM) was added 3 min after the start of incubation.

Inhibition of Ca$^{2+}$/H₁₁₀₀¹-dependent dephosphorylation at MAP
kinase-dependent P-sites 4/5 and 6 in synaptosomes is completely blocked
by the calcineurin inhibitor cyclosporin A (CsA). Synaptosomes were
incubated for 10 min in the presence of 0.2 mM EGTA and in the absence
or presence of cyclosporin A (1 μM) and then depolarized with 4-AP just
before the addition of 1.2 mM CaCl₂. Control samples were collected
before the addition of 4-AP. The effect of Ca$^{2+}$/H₁₁₀₀¹ entry was then followed
at 30 sec, 1 min, and 10 min using SDS-PAGE and immunoblotting with
P-site specific antibodies. A, Ca$^{2+}$/H₁₁₀₀¹-dependent dephosphorylation of P-site

B, Ca$^{2+}$/H₁₁₀₀¹-dependent dephosphorylation of P-site 6 resulted in a decrease to 77 ± 2.8% of control (mean ± SEM; n = 4), 1 min after depolarization by 4-AP (control). This effect was com-
pletely inhibited in the presence of cyclosporin A, resulting in a small
increase to 108 ± 8.1% of control (mean ± SEM; n = 4), 1 min after
depolarization by 4-AP (CsA).

C, Ca$^{2+}$/H₁₁₀₀¹-dependent phosphorylation of P-site 1 resulted in an increase to 155.5 ± 9.4% of control (mean ± SEM; n = 4), 1 min after depolarization by 4-AP (control). No significant effect (144 ± 9.6% of control; mean ± SEM; n = 4) was observed at 1 min in the presence of CsA.

D, Ca$^{2+}$/H₁₁₀₀¹-dependent phosphorylation of P-site 3 resulted in an increase to 1391 ± 305% of control (mean ± SEM; n = 4), 1 min after depolarization by 4-AP (control). No significant effect (1024 ± 165% of control; mean ± SEM; n = 4) was observed at 1 min in the presence of CsA.

MAP kinase was observed in the presence of okadaic acid as compared with controls (Fig. 4B). This effect likely reflected a direct inhibition of PP2A-dependent dephosphorylation/deactivation of MAP kinases (Alessi et al., 1995; Chajry et al., 1996).

The ability of calcineurin to dephosphorylate various P-sites in synapsin I was tested using a specific inhibitor, cyclosporin A (1 μM). However, given previous reports of increased Ca\(^{2+}\) channel activity in the presence of calcineurin inhibitors (Sihra et al., 1995; Lukyanetz et al., 1998; Burley and Sihra, 2000), one potential complication in this experimental design is that an increase in CaM kinase-dependent phosphorylation of synapsin I may result from an increase in Ca\(^{2+}\) influx in the presence of cyclosporin A. To attempt to obviate this possibility, we incubated synaptosomes for 10 min in the presence of 0.2 mM EGTA in the absence or presence of cyclosporin A, and depolarized the synaptosomes with 4-AP before the addition of 1.2 mM CaCl\(_2\). Control samples were collected before the addition of 4-AP/Ca\(^{2+}\). The phosphorylation of synapsin I at specific sites was then determined 30 sec, 1 min, and 10 min after the addition of Ca\(^{2+}\) by immunoblotting with P-site-specific antibodies. Inhibition of calcineurin activity by cyclosporin A led to complete inhibition of Ca\(^{2+}\)-dependent dephosphorylation of P-site 4/5 (Fig. 5A) and P-site 6 (Fig. 5B) with little or no effect on phosphorylation of P-site 1 (Fig. 5C) and P-site 3 (Fig. 5D). Cyclosporin A had no effect on basal levels of phosphorylation of synapsin I at specific sites before depolarization and Ca\(^{2+}\) entry.

**Functional correlation of Ca\(^{2+}\)-dependent dephosphorylation of synapsin I with the release of glutamate**

To examine the possible functional significance of sustained high levels of phosphorylation of P-sites 4/5 and 6 in synapsin I produced by inhibition of calcineurin activity, we measured ionomycin-triggered glutamate release in the presence or absence of cyclosporin A using an on-line fluorometric assay (Nicholls and Sihra, 1986). Ionomycin causes a direct increase in intrasynaptosomal Ca\(^{2+}\) levels and triggers release of neurotransmitter without depolarization and Ca\(^{2+}\) channel activation (Sihra et al., 1992), therefore allowing us to detect only those modulatory influences directly affecting synaptic vesicle trafficking and exocytosis.

Incubations were performed at 37°C in the presence of CoCl\(_2\) (10 μM) and CdCl\(_2\) (10 μM) to completely block the activity of Ca\(^{2+}\) channels (Vickroy et al., 1992). The inhibition of Ca\(^{2+}\)-channel activity by CoCl\(_2\) and CdCl\(_2\) was complete and resulted in an abrogation of Ca\(^{2+}\)-dependent glutamate release evoked by 30 mM KCl (Fig. 6, inset). Cyclosporin A (1 μM) was applied for 10 min, after which glutamate release was triggered by the addition of ionomycin in the presence of 1 mM Ca\(^{2+}\). Ionomycin caused a Ca\(^{2+}\)-dependent glutamate release of 11.5 ± 1.5 nmol/mg after 4 min (n = 3). In the presence of cyclosporin A, ionomycin-induced release of glutamate was potentiated by 35 ± 9% (n = 3; p < 0.05; Student’s paired t test) to 15.2 ± 1.2 nmol/mg after 4 min (Fig. 6).

**DISCUSSION**

We report here that nerve terminal stimulation and Ca\(^{2+}\) influx regulate the state of phosphorylation of the synaptic vesicle-associated protein synapsin I via two opposing mechanisms: (1) Ca\(^{2+}\)-regulated kinase activities increase phosphorylation of synapsin I P-sites 1, 2, and 3, and (2) Ca\(^{2+}\)-regulated phosphatase activity decreases phosphorylation of P-sites 4, 5, and 6 (Scheme 2). These effects occurred with similarly fast kinetics and were consistent with the influx of Ca\(^{2+}\)-activating CaM kinases I and II and calcineurin, respectively. We have also identified PP2A as the phosphatase that downregulates synapsin phosphorylation at P-sites 1, 2, and 3. In intact nerve terminals, calcineurin plays a role in regulating the phosphorylation state of synapsin I and concomitantly modulates glutamate release. Given the established importance of synapsin I-dependent regulation of synaptic vesicle trafficking from reserve pools of synaptic vesicles (Hilfiker et al., 1999), calcineurin appears to play a key role in activity-dependent modulation of nerve terminal function.

**Nerve terminal phosphatase activities**

Serine/threonine phosphatases have been extensively studied, but few studies have directly addressed their role in nerve terminal function (Sihra et al., 1992, 1995; Nichols et al., 1994; Steiner et al., 1996). Here we have characterized the role of the major serine–threonine phosphatases by examining their activity against a nerve terminal-specific substrate, synapsin I, both in *vitro* (using purified components) and in *situ* (within nerve terminals, using phosphorylation-state specific antibodies to various P-sites of synapsin I). Our results point to the presence and tonic activity of PP2A that limits the basal phosphorylation state of synapsin at P-sites 1, 2, and 3. After stimulation of nerve terminals, Ca\(^{2+}\) influx causes the rapid activation of calcineurin, to effect the dephosphorylation of synapsin at P-sites 4, 5, and 6. In contrast, none of the synapsin I P-sites appeared to be a good substrates for PPI, reflecting the primarily postsynaptic localiza-
tion of this phosphatase and its established association with dendritic structures (Allen et al., 1997). Taken together, these data demonstrate that PP2Ac is the most likely phosphatase involved in the regulation of phosphorylation state of P-sites 1, 2, and 3 of synapsin I. However, calcineurin is the most likely phosphatase involved in regulation of the phosphorylation state of P-sites 4, 5, and 6, with a significantly lower activity for other synapsin I phospho-forms. Although both PP2A and calcineurin are ubiquitous enzymes, the latter is particularly enriched in the brain (Usuda et al., 1996) where it has been implicated in several forms of synaptic plasticity (Mansuy et al., 1998; Winder et al., 1998). Specific effects of calcineurin on presynaptic function may well be partly responsible for these effects on synaptic plasticity.

**Phosphorylation-dephosphorylation of synapsin P-sites 1, 2, and 3**

Synapsins interact dynamically with synaptic vesicles and actin *in vitro* and *in situ*. Phosphorylation of synapsin I at sites 2 and 3 by CaM kinase II results in a profound change in its conformation and binding to synaptic vesicles (Jovanovic et al., 1996). Synapsin I bundles F-actin in a phosphorylation-dependent manner. Nature 326:704–706.

The fast rise in phosphorylation of P-sites 1, 2, and 3, as well as the relatively slow dephosphorylation of these sites during prolonged stimulation of nerve terminals (Fig. 3A,B) (5 and 10 min), are in agreement with our *in vitro* dephosphorylation data and point to regulation of these sites by a prolonged increase in CaM kinase activity concomitant with a tonic, CaMKII results in a profound change in its conformation and binding to synaptic vesicles (Jovanovic et al., 1996). Synapsin I bundles F-actin in a phosphorylation-dependent manner. Nature 326:704–706.

The basal activity of MAP kinases (Fig. 1C), together with their CaMKII results in a profound change in its conformation and binding to synaptic vesicles (Jovanovic et al., 1996). Synapsin I bundles F-actin in a phosphorylation-dependent manner. Nature 326:704–706.

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