Tyrosine phosphorylation of synapsin I by Src regulates synaptic-vesicle trafficking

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Summary

Synapsins are synaptic vesicle (SV)-associated phosphoproteins involved in the regulation of neurotransmitter release. Synapsins reversibly tether SVs to the cytoskeleton and their phosphorylation by serine/threonine kinases increases SV availability for exocytosis by impairing their association with SVs and/or actin. We recently showed that synapsin I, through SH3- or SH2-mediated interactions, activates Src and is phosphorylated by the same kinase at Tyr301. Here, we demonstrate that, in contrast to serine phosphorylation, Src-mediated tyrosine phosphorylation of synapsin I increases its binding to SVs and actin, and increases the formation of synapsin dimers, which are both potentially involved in SV clustering. Synapsin I phosphorylation by Src affected SV dynamics and was physiologically regulated in brain slices in response to depolarization. Expression of the non-phosphorylatable (Y301F) synapsin I mutant in synapsin-I-knockout neurons increased the sizes of the readily releasable and recycling pools of SVs with respect to the wild-type form, which is consistent with an increased availability of recycled SVs for exocytosis. The data provide a mechanism for the effects of Src on SV trafficking and indicate that tyrosine phosphorylation of synapsins, unlike serine phosphorylation, stimulates the reclustering of recycled SVs and their recruitment to the reserve pool.

Key words: Neurotransmitter release, Tyrosine kinases, Exo-endocytosis, Synaptic plasticity, Neurotrophins, Actin

Introduction

Tyrosine kinases are known to have a key role in neuron physiology, both during development and in mature neurons. The levels of protein tyrosine kinases and tyrosine phosphatases in the nervous system are very high, and many growth factors and cytokines exert their biological actions by directly or indirectly affecting the activity of receptor-associated or non-receptor tyrosine kinases. Although receptor tyrosine kinases activated by neurotrophins have been extensively studied, comparatively little is known about non-receptor tyrosine kinases in neurons in spite of their relative abundance. The product of the cellular homologue of the viral src oncogene pp60

© (Src) represents the prototype of non-receptor protein tyrosine kinases. Src is expressed by virtually all cells, and very high expression levels are found in cells specialized for regulated secretion, particularly neurons, suggesting a role in synaptic transmission and plasticity (Purcell and Carew, 2003; Kalia et al., 2004; Ingleby, 2008).

Although Src has been convincingly implicated in the regulation of post-synaptic receptor sensitivity to neurotransmitters through phosphorylation and modulation of glutamate and nicotinic acetylcholine receptors (Hayashi and Huganir, 2004; Charpentier et al., 2005; Salter and Kalia, 2004), its role and the signaling pathways that regulate its activity at the presynaptic level have not been clearly identified. However, several synaptic-vesicle (SV) substrates for Src kinases have been demonstrated, including synaptophysin, synaptogyrin, cellugyrin and synapsins. High levels of Src are also associated with SVs and the presynaptic membrane, with a four- to fivefold enrichment in purified SVs compared with brain homogenate (Pang et al., 1988; Barnekow et al., 1990; Onofri et al., 1997; Onofri et al., 2007; Stenius et al., 1995; Janz and Südhof, 1998).

A number of recent studies implicate Src kinases in the regulation of presynaptic physiology. High-frequency synaptic stimulation, epileptiform activity and spatial learning trials activate Src kinases and promote their association with interacting and/or substrate proteins, including synapsin I (Lauri et al., 2000; Sanna et al., 2000; Zhao et al., 2000). Moreover, studies using specific Src inhibitors indicate that Src-family kinases directly modulate neurotransmitter release by interfering with activity-dependent Ca2+-entry, actin dynamics and SV exocytosis. Inhibition of Src kinase by PP2 was found to enhance evoked neurotransmitter release from PC12 cells and primary neurons, whereas the expression of constitutively active Src, but not of a kinase-dead mutant, suppressed Ca2+-dependent release (Ohnishi et al., 2001). These results were subsequently confirmed in rat brain synaptosomes and pancreatic β-cells, in which the Src inhibitors PP1 and PP2 increased glutamate release evoked by depolarization and Ca2+-induced insulin secretion, respectively (Balwani et al., 2006; Cheng et al., 2007). However, other studies carried out in synaptosomes reported a decrease, rather than an increase, in glutamate release evoked by ionomycin or depolarization after Src inhibition by PP2 (Wang, 2003; Shyu et al., 2005).

One of the mechanisms by which Src regulates neurotransmitter release in neurons is by modifying, through phosphorylation, the activity of proteins involved in the regulation of exocytosis and SV trafficking, such as the synapsins. The synapsins are major SV-
associated phosphoproteins which are at the convergence of multiple serine/threonine kinase pathways, including those linked to the activation of cAMP-dependent protein kinase (PKA), Ca2+-calmodulin-dependent protein kinases (CaMKI, CAMKII and CAMKIV) and mitogen-activated protein kinase/Erk (MAPK/Erk) (for a review, see Cesca et al., 2010). Phosphorylation on serine residues promotes the dissociation of the synapsins from SVs and/or the actin meshwork, followed by their cytosolic diffusion and the transition of SVs from the reserve pool (RP) to the readily releasable pool (RRP) to foster exocytosis (Chi et al., 2001; Chi et al., 2003; Menegon et al., 2006) (for a review, see Cesca et al., 2010). We have recently shown that synapsins are excellent Src substrates and are phosphorylated at a major and highly conserved tyrosine residue located in domain C (Onofri et al., 2007), a region that is involved in binding to both SVs and actin.

We thus considered the possibility that tyrosine phosphorylation is involved in the regulation of neurotransmitter release by affecting SV mobility. To investigate this, we studied the physiological effects and the regulation of Src phosphorylation of synapsin I. We found that phosphorylation by Src stimulates the oligomerization of synapsin I and increases its association with SVs and actin filaments, which are opposite effects to those seen with serine phosphorylation. Moreover, we found that phosphorylation of synapsin I on tyrosine is physiologically regulated by depolarization and stimulates the reclustering of recycled SVs and their recruitment to the RP during neuronal activity.

Results
Tyrosine phosphorylation increases the interactions of synapsin I with synaptic vesicles and actin
Specific antibodies against phosphorylated Tyr301 were generated by immunizing rabbits with a synapsin I 10-mer peptide encompassing the major phosphorylated tyrosine (Onofri et al., 2007). The affinity-purified antibodies specifically recognized purified synapsin I that was phosphorylated in vitro by Src in a concentration-dependent fashion (Fig. 1A), but they did not detect dephosphorylated synapsin I or synapsin I phosphorylated on distinct serine residues by either PKA (site 1), CaMKII (sites 2 and 3) or MAPK/Erk (sites 4, 5 and 6) (Fig. 1B). The antibodies also recognized tyrosine-phosphorylated synapsin I in purified SVs (Fig. 1C, upper panel) and brain slices (see below). In purified untreated SVs (USVs), the antibodies revealed a low constitutive level of synapsin I tyrosine phosphorylation by endogenous SV Src, which was markedly increased by the addition of ATP and virtually disappeared when synapsin I was quantitatively dissociated from SVs by dilution in high-salt medium (SSVs; Fig. 1C, lower panel).

Serine phosphorylation of synapsin I is known to negatively modulate its interactions with SVs and/or actin (Cesca et al., 2010). Thus, it was of interest to analyze whether phosphorylation of synapsin I on tyrosine is also effective in altering these interactions. We first studied the phosphorylation dependence of the dissociation of synapsin I from SVs purified under low ionic strength, which maintain their saturation with endogenous synapsin I during the purification procedure and contain a significant amount of endogenous catalytically active Src (Huttner et al., 1983; Onofri et al., 1997). Purified native SVs were incubated in the absence or presence of ATP or ATP plus Src to induce a progressively higher tyrosine phosphorylation of synapsin I (Fig. 2A) and synapsin I dissociation was triggered by dilution under either low (40 mM NaCl) or high (200 mM NaCl) ionic strength conditions. Interestingly, tyrosine phosphorylation counteracted the dissociation of synapsin I in a concentration-dependent fashion and the effect was particularly evident at 200 mM NaCl, a condition known to achieve a quantitative dissociation of synapsin I from the SV membrane under basal conditions (Fig. 2A,B). Next, we analyzed the binding of purified synapsin I, which had been phosphorylated to low (0.1 mol/mol) stoichiometry at Tyr301 by Src, to purified synapsin-I-depleted SVs. Quantitative immunoblotting and 32P counting were used to follow the total (mainly dephosphorylated) synapsin I and the tyrosine-phosphorylated synapsin I, respectively, in the same samples. In agreement with the results of the dissociation experiments, tyrosine-phosphorylated synapsin I bound more avidly to SVs than the dephosphorylated form, as demonstrated by the significantly higher ratios of bound to total synapsin I at intermediate and high concentrations of synapsin I (Fig. 2C).

![Fig. 1. Synapsin I is phosphorylated by purified and synaptic-vesicle-associated Src as detected by phosphospecific antibodies against Tyr301-P synapsin I.](image-url)

- **A**: Purified bovine synapsin I (0.2-0.4 μg) was incubated under tyrosine-phosphorylation conditions in the absence or presence of ATP (100 μM) and Src at 30°C for 2 hours, separated by SDS-PAGE and analyzed by immunoblotting with anti-synapsin-I antibodies (10.22 monoclonal; SynI) or with the Tyr301-P synapsin I phosphospecific antibody (0.3 μg/ml; PTyr-SynI). Molecular size markers in kDa are indicated on the left.
- **B**: Specificity of the phosphospecific antibodies against synapsin I. Equal amounts (100 ng/sample) of dephosphorylated synapsin I (DP-SynI) or synapsin I phosphorylated in vitro by the indicated protein kinases (PKA, CaMKII, MAPK/Erk or Src, respectively) were subjected to immunoblotting with anti-synapsin-I antibodies (10.22 monoclonal; SynI) or phosphospecific antibodies specific for site 1 (1P-SynI; PKA phosphorylation), site 3 (3P-SynI, CaMKII phosphorylation), sites 4,5,6 (4,5P-SynI; MAPK/Erk phosphorylation) or Tyr301 (0.3 μg/ml; PTyr-SynI; Src phosphorylation). Immunoblotting with an anti-phosphotyrosine antibody is also shown (PTyr).
- **C**: Purified untreated SVs (USV) or SVs depleted of endogenous synapsin I (SSV) were incubated under tyrosine-phosphorylation conditions in the absence or presence of ATP (100 μM) at 30°C for 40 minutes. Proteins were separated by SDS-PAGE and analyzed by immunoblotting as described in A. In the lower panel, the endogenous phosphorylation of synapsin I at Tyr301 on SVs was quantitatively analyzed by densitometry of the fluorograms and expressed as mean ± s.e.m. (n=4). **P<0.01, Dunnett’s multiple comparison test vs control.
Similar studies were performed to assess the effects of tyrosine phosphorylation on the interactions of synapsin I with actin filaments. Purified synapsin was phosphorylated by Src as described above and subsequently incubated with preformed actin filaments. After separation of free and actin-filament-bound synapsin I by high-speed centrifugation, dephosphorylated and tyrosine-phosphorylated synapsin I were analyzed by immunoblotting with anti-synapsin-I antibodies and 32P counting in both pellet and supernatant fractions. As observed for SV binding, tyrosine-phosphorylated synapsin I bound actin filaments more avidly with a significantly higher ratio (15.02±3.63; bound to free ratio) than that measured with dephosphorylated synapsin I (2.27±0.48; *P<0.01, Student’s t-test) (Fig. 3).

Tyrosine phosphorylation affects synapsin I oligomerization

Synapsin I is known to form homo-oligomers as well as hetero-oligomers with other synapsin isoforms – a phenomenon that involves domains C and E (Esser et al., 1998; Hosaka and Südhof, 1999; Monaldi et al., 2010). To quantitatively evaluate the effects of tyrosine phosphorylation on synapsin I homo-oligomerization, purified synapsin I was incubated in the presence of ATP and/or Src and subjected to chemical crosslinking with disuccinimydil...
that synapsin I was phosphorylated only under the latter condition, with detectable phosphate incorporation associated with the synapsin I monomer and dimer (Fig. 4A, right panel). The apparently lower phosphorylation stoichiometry of the synapsin I dimer with respect to the monomer could be due to the fact that antibodies recognizing distinct sites experience different accessibility to the respective epitope, once the dimer is crosslinked. In this respect, although the anti-synapsin-I antibody used recognizes domain D (which is not involved in self-association), the anti-phosphotyrosine epitope is in close proximity to the dimerization plane. Moreover, ATP itself induces an increase in the amount of synapsin I dimer independently of the phosphorylation state, so that in the presence of ATP and Src, only a fraction of the total dimer is expected to be phosphorylated.

**Depolarization increases tyrosine phosphorylation of synapsin I in brain slices**

The above-reported in vitro experiments demonstrate that phosphate incorporation at Tyr301 has a significant role in the regulation of synapsin I interactions. To ascertain whether this also occurs in intact nerve terminals, we first addressed the question of whether the phosphorylation of synapsin I by Src is physiologically regulated by electrical activity in acute cortical slices. High K+-induced depolarization of acute cortical slices promoted a clear-cut increase in phosphorylation of synapsin I at Tyr301 with respect to resting conditions (Fig. 5A). The increase in the anti-Tyr301-P immunoreactivity was dependent on the presence of extracellular Ca²⁺ and was virtually abolished by pretreatment of slices with the Src inhibitor PP2 (20 μM; Fig. 5B). The depolarization-evoked increase in the tyrosine phosphorylation of synapsin I was accompanied by a concomitant, marked decrease in the phosphorylation state of the MAPK/Erk sites 4 and 5, as a consequence of calcineurin activation (Jovanovic et al., 1996; Jovanovic et al., 2001) (Fig. 5A,B). We also examined the time-course of synapsin I tyrosine phosphorylation in response to a 2 minute depolarization pulse with 40 mM KCl, followed by repolarization, and compared it with that of MAPK/Erk phosphorylation of synapsin I. Phosphorylation at Tyr301 exhibited a peak value within 2 minutes of the onset of depolarization and remained above basal levels for over 30 minutes. Synapsin I phosphorylation at the MAPK/Erk sites sharply decreased during depolarization and exhibited a sustained increase above baseline during the repolarization period, which was similar to that observed for Tyr301 phosphorylation (Fig. 5C). Thus, the states of phosphorylation of the MAPK/Erk and Src sites of synapsin I exhibited opposite changes in the early phase of depolarization, whereas they were both increased in the delayed phase that follows repolarization.

**The non-phosphorylatable tyrosine mutant of synapsin I boosts SV trafficking**

The observation that synapsin I tyrosine phosphorylation by Src in nerve terminals is activity dependent suggests that it is physiologically important. To investigate the role of phosphorylation of synapsin I at Tyr301 in the regulation of SV exo-endocytosis during synaptic activity, we performed site-directed mutagenesis of mouse synapsin Ia by substituting the non-phosphorylatable residue Phe for Tyr301, yielding a tyrosine dephosphomimetic synapsin I mutant (Y301F-SynI). The Cherry-tagged chimeras of wild type (WT) or Y301F-SynI were expressed in intact nerve terminals, we first addressed the question of whether the phosphorylation of synapsin I by Src is physiologically regulated by electrical activity in acute cortical slices. High K+-induced depolarization of acute cortical slices promoted a clear-cut increase in phosphorylation of synapsin I at Tyr301 with respect to resting conditions (Fig. 5A). The increase in the anti-Tyr301-P immunoreactivity was dependent on the presence of extracellular Ca²⁺ and was virtually abolished by pretreatment of slices with the Src inhibitor PP2 (20 μM; Fig. 5B). The depolarization-evoked increase in the tyrosine phosphorylation of synapsin I was accompanied by a concomitant, marked decrease in the phosphorylation state of the MAPK/Erk sites 4 and 5, as a consequence of calcineurin activation (Jovanovic et al., 1996; Jovanovic et al., 2001) (Fig. 5A,B). We also examined the time-course of synapsin I tyrosine phosphorylation in response to a 2 minute depolarization pulse with 40 mM KCl, followed by repolarization, and compared it with that of MAPK/Erk phosphorylation of synapsin I. Phosphorylation at Tyr301 exhibited a peak value within 2 minutes of the onset of depolarization and remained above basal levels for over 30 minutes. Synapsin I phosphorylation at the MAPK/Erk sites sharply decreased during depolarization and exhibited a sustained increase above baseline during the repolarization period, which was similar to that observed for Tyr301 phosphorylation (Fig. 5C). Thus, the states of phosphorylation of the MAPK/Erk and Src sites of synapsin I exhibited opposite changes in the early phase of depolarization, whereas they were both increased in the delayed phase that follows repolarization.

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Fig. 5. Depolarization increases tyrosine phosphorylation of synapsin I in acute cortical slices. (A) Acute cortical slices were incubated under resting conditions or depolarized by 40 mM KCl for 2 minutes in the presence of extracellular Ca++. Slice homogenates were subjected to SDS-PAGE and quantitative immunoblotting with anti-synapsin-I antibodies (SynI), anti-Tyr301-P synapsin I antibodies (PTyr-SynI) or antibodies against MAPK/Erk-phosphorylated synapsin I (sites 4,5; 4,5P-SynI). A representative experiment is shown. Arrows point to the synapsin Ia and synapsin Ib doublet. (B) Cortical slices were incubated as described in A in the absence (resting) or presence of KCl (40 mM), EGTA (2 mM) or PP2 (20 μM). The extent of depolarization-induced synapsin phosphorylation by Src and MAPK/Erk was calculated as the immunoreactivity ratio between phosphorylated and total synapsin I and expressed as percentage change with respect to the ratio observed under resting conditions (means ± s.e.m. of six independent experiments). **P<0.01; Dunnett’s multiple comparison test vs resting. (C) Time-course of synapsin I phosphorylation at Tyr301 (PTyr-SynI; closed symbols) or at sites 4,5 (4,5P-SynI; open symbols) following a 2 minute depolarization pulse with 40 mM KCl (black horizontal bar) and the subsequent repolarization period. Synapsin I phosphorylation, measured as described in (B), is expressed as percentage change with respect to the resting condition as a function of time after the onset of the depolarizing pulse (means ± s.e.m. of four independent experiments).
The demonstration that synapsin I is an excellent Src substrate and is phosphorylated by Src at Tyr301 in the highly conserved domain C (Onofri et al., 2007) prompted us to investigate whether tyrosine phosphorylation modulated the molecular interactions of synapsin I and had an effect on presynaptic function. The highly conserved domain C is a region of key importance in synapsin I function. It contains a major actin-binding site (mapped to Syn223-360) (Bähler et al., 1989), a major SV-binding site, including a sequence that penetrates the phospholipid bilayer (mapped to Syn278-327) (Benfenati et al., 1989a; Benfenati et al., 1989b; Cheetham et al., 2001) and dimerization-tetramerization sequences (Esser et al., 1998; Brautigam et al., 2004) involved in synapsin oligomerization (Hosaka and Südhof, 1999). Domain C also has structural similarity with ATP transferases and binds ATP (Esser et al., 1998). The Src site is the first phosphorylation site identified in synapsin I domain C and insertion of a negative phosphate group in this very hydrophobic and highly charged domain could affect these interactions through changes in the secondary structure of the molecule and/or in the electrostatic environment. Unfortunately,
owing to its very poor solubility, there are no functional data regarding the effects of the domain C sequence encompassing the tyrosine phosphorylation site (Hilfiker et al., 2005).

The possibility that tyrosine phosphorylation affects the molecular interactions of synapsin I was addressed in SV-binding and actin-binding assays. Tyrosine phosphorylation was found to increase the association of synapsin I with SVs and actin filaments, and also induced a shift in the proportion of synapsin I tetramers and dimers. The effect on SV interactions was present on both the binding of endogenous synapsin I and the in vitro reassociation of purified synapsin I to synapsin-I-depleted SVs, suggesting an increased affinity for the SV membrane. Notably, this effect is opposite to the inhibitory effect on SV binding reported for PKA/CaMKI and CaMKII phosphorylation of synapsin I (Schiebler et al., 1986; Benfenati et al., 1989a; Benfenati et al., 1992a; Hosaka et al., 1999). Tyr301 phosphorylation of synapsin I also increased its F-actin-binding activity, as opposed to the inhibition of the actin-synapsin-I interaction reported for PKA/CaMKI, CaMKII and MAPK/Erk phosphorylation of synapsin I (Bähler and Greengard, 1987; Benfenati et al., 1992b; Valtorta et al., 1992; Nielander et al., 1997; Jovanovic et al., 1996).

The crystal structure of the synapsin I domain C revealed that it forms a tight dimer (Esser et al., 1998) along a plane that does not overlap with the SV-membrane-binding domains of synapsin I, thus making it possible that synapsin I dimers mediate SV clustering (Benfenati et al., 1993; Cheetham et al., 2001). Moreover, dimers of the N-terminal region of synapsin I (domains A-C) associate to form tetramers, which are stabilized by Ca\(^{2+}\)-ATP binding to the highly conserved ATP-binding site of domain C (Brautigam et al., 2004). Tetramers are characterized by a ‘multifunctional flexible loop’ corresponding to Syn130-343, which participates in the tetramer interface and contacts bound ATP (Brautigam et al., 2004). We confirmed that micromolar concentrations of ATP also induce the formation of synapsin I tetramers in the presence of divalent cations other than Ca\(^{2+}\), suggesting that the tetramer could be the major oligomerization state of synapsin I within nerve terminals. Interestingly, phosphorylation of Tyr301 shifts the equilibrium from tetramer to dimer, probably by interfering with the nearby residues involved in contacts between dimers to form tetramers (Syn290-299) or with the multifunctional loop. An increase in synapsin I dimerization could have a potential effect on SV trafficking if, as it is currently believed (Cheetham et al., 2001; Cesca et al., 2010), synapsin I dimers are the preferred oligomer for crosslinking adjacent SVs to form clusters. Interestingly, it has recently been reported that phosphorylation of synapsin I by MAPK/Erk decreases its ability to heterodimerize with synapsin IIA (Ryoo and Augustine, 2009).

Since binding of synapsin I to SVs and actin filaments, and synapsin I dimerization are believed to collectively participate in the formation of SV clusters and their anchorage to the actin cytoskeleton, Src phosphorylation of synapsin I might favor the recruitment of SVs to the RP and decrease the transition of SVs from the RP to the RRP. This hypothesis is consistent with the decrease in evoked release reported after Src inactivation in neurons and isolated nerve terminals (Ohnishi et al., 2001; Baldwin et al., 2006; Cheng et al., 2007). This possibility was tested experimentally by studying SV pools and trafficking in Syn1-KO neurons expressing the dephosphomimetic synapsin I mutant. The increase in the response to a short AP train in neurons expressing the dephosphomimetic mutant points to a higher number of SVs in the RRP. A similar increase in the response to the long AP train was evident only under conditions in which endocytosis was blocked, suggesting that the expression of this mutant causes an increased number of SVs to be released by the same train as a consequence of an increased and sustained SV supply to the RRP. Such increased supply of SVs to the RRP can be compensated, under normal conditions, by an increased rate of endocytosis. Altogether, the live imaging data indicate that, in the presence of the synapsin I dephosphomimetic mutant, SVs are more easily released in response to the electrical stimulation, probably as a consequence of a lack of sequestration of endocytosed SVs in the RP and of a more dynamic recycling pool of SVs, which promptly refills the depleted RRP.

The present data, together with the previously reported information on the other synapsin I phosphorylation sites, suggest a model in which serine phosphorylation boosts release, whereas tyrosine phosphorylation favors recovery and reconstitution of the SV stores. Here, we have shown that, similarly to serine phosphorylation, tyrosine phosphorylation of synapsin I is also physiologically regulated and is activated by depolarization in a Ca\(^{2+}\)-dependent fashion. This means that activity-dependent Ca\(^{2+}\)-entry is ultimately responsible for Src activation on SVs. Phosphorylation of SV tyrosine substrates by Src has been shown to be an intravesicular event (Pang et al., 1988) and therefore synapsin I molecules can be phosphorylated by Src as soon as they reassociate with SVs after stimulation and during the subsequent repolarization period. The sustained increase of synapsin I tyrosine phosphorylation after depolarization is fully compatible with this model and suggests a function in orienting the fate of recycled SVs.

But, how are serine and tyrosine phosphorylation of synapsin I dynamically interconnected during nerve-terminal activity? Synapsin I is composed of a mosaic of phosphorylation sites that are independently regulated by kinases and phosphatases, which, under certain functional conditions, can be co-activated. Thus, the overall effect of a stimulus might depend on the selectivity of activation of the signal-transduction pathway, and, in the case of simultaneous activation of parallel pathways (as occurs during depolarization), by specific enzyme activation. Upon depolarization-evoked Ca\(^{2+}\) influx, CaMKs, PKA, MAPK/Erk, calcineurin and Src all become activated, albeit at distinct rates. This elicits a prompt phosphorylation of PKA and CaMK sites and a concomitant calcineurin-mediated dephosphorylation of MAPK/Erk sites (Cesca et al., 2010). In this picture, the slower Src activation might become significant in a later phase of stimulation, in which, after endocytosis, the RRP and RP are refilled.

What could be the significance of tyrosine phosphorylation of synapsin I in nerve-terminal function? The binding of SVs to actin is important for the maintenance and reconstitution of the reserve pool of SVs and as a protection mechanism that prevents excessive reserve-pool depletion during sustained intense electrical activity (Brodin et al., 1997; Rizzoli and Betz, 2005). The synapsin-I-mediated attachment of SVs to actin filaments, as well as the stimulation of actin polymerization induced by synapsin I, are thought to also have a role in SV recycling at perisynaptic zones, where a pool of synapsin I bound to the actin-rich cytomatrix reassociates with SVs after endocytosis and clathrin uncoating (Bloom et al., 2003). Moreover, regulation of SV availability for exocytosis is involved in synaptic-plasticity paradigms, in which the synapsin proteins are thought to have an important role (Cesca et al., 2010). The present data indicate that the synapsins control SV availability for exocytosis by a push-pull mechanism acting on
the rate constants of SV clustering and cytoskeleton attachment. Serine phosphorylation of synapsin I promotes unclustering and release of SVs from the cytoskeletal constraint, whereas tyrosine phosphorylation by Src might promote the opposite reactions. This configures a ‘yin-yang’ control of serine and tyrosine phosphorylation on SV trafficking, which further emphasizes the central role of synapsin I at the convergence and integration of numerous signal-transduction pathways activated by extracellular messengers and electrical activity.

Materials and Methods

Materials

- [γ-32P]ATP (>3,000 Ci/mmol), protein-G- Sepharose, the Amersham ECL Plus western blotting reagent and Kodak X-Omat films were from GE Healthcare (Milano, Italy).
- Antibodies against synapsin and synaptophysin were raised in our laboratory. Phosphorylation-state-specific antibodies recognizing phosphorylated sites 1, 3 and 4 of synapsin I (Jovanovic et al., 1996; Jovanovic et al., 2001) were generated in Paul Greengard’s laboratory (The Rockefeller University, New York, NY). Human recombinant Src kinase (specific activity 1 U/ml) was purchased from Upstate Biotechnology. The crosslinking agent DSS was from Pierce Biotechnology (IL). Phosphate buffer of high ionic strength (H2PO4/NaH2PO4 buffer) containing phenylmethylsulfonyl fluoride (0.1 mM), pepstatin (1 µg/ml) and leupeptin (1 µg/ml) obtained from Sigma. Tetrodotoxin was from Tocris (Bristol, UK) and 4-amino-5-(4-chlorophenyl)-7-[(2-hydroxyethyl)amino]-3(2H)-pyridazinone (PP2) from Calbiochem-Novabiochem (Germany). Synapsin-I KO mice were generated by homologous recombination (Onofri et al., 1999). Offspring of littermates of C57/B6l wild type and fully backcrossed homozygous Synl-KO mice were used throughout. All animal procedures were approved by the Animal Care Committee of the University of Genova and by the Italian Ministry of Health.

Generation of phosphorylation-state-specific antibodies

Antibodies recognizing synapsin I phosphorylated by Src at Tyr301 were raised by using the 10-mer synapsin I peptide Cys-TKTYPATAEP-amide encompassing the major Src phosphorylation site (Y301) (Onofri et al., 2007) as antigen, following the previously described procedure (Czernecki et al., 1991) (PhosphoSpotSolutions, Aurora, CO). Hyperimmune sera were depleted of dephosphospecific antibodies through a dephosphopeptide-CH-Sepharose column and passed on a phosphopeptide-CH-Sepharose column for the purification of phosphopeptide-specific antibodies.

Protein purification, phosphorylation and subcellular fractionation

Synapsin I was purified from bovine brain (Bährler and Greengard, 1987). Purified synapsin I was phosphorylated in vitro on specific sites by PKA (site 1), CaMKII (site 2) and PKC (sites 4,5) (Bähler and Greengard, 1987). Phosphorylation of purified synapsin I by Src was performed by incubation in phosphorylation buffer (20 mM HEPES, pH 7.4, 10 mM MnCl2, 2 mM EGTA, 0.4 mM Na3VO4, 10 mM magnesium acetate, 1 mM ATP, 5 mM MgCl2. Slices were incubated for 1 hour at 37°C with moderate agitation, under a humidified atmosphere of O2:CO2 (95:5 v/v) for 30 minutes at 4°C) for recovery of total F-actin (Bähler and Greengard, 1987). Purification of SVs was carried out through the step of controlled-pore glass chromatography (Huttner et al., 1983).

Synaptic-vesicle assays

For the dissociation of endogenously bound synapsin I, purified native SVs (40 µg/sample) were incubated for 40 minutes at 30°C under tyrosine phosphorylation conditions in the absence of ATP or in the presence of ATP either alone or supplemented with purified Src (3-6 µg/sample; 1-2 mM final concentration) under either low (0.3 M glycine, 40 mM NaCl, 5 mM HEPES, pH 7.4) or high (0.3 M glycine, 200 mM NaCl, 5 mM HEPES, pH 7.4) ionic strength conditions in the presence of protease inhibitors. After incubation for 1 hour at 37°C, SVs were recovered by high speed centrifugation (400,000 g for 30 minutes) and the amounts of bound synapsin I were determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as reported below. The binding of purified synapsin I to synapsin-I-depleted SVs was performed as previously described (Schiebler et al., 1986). Synapsin-I-depleted SVs (10 µg/sample) were incubated for 1 hour at 0°C with increasing concentrations (25-200 nM) of synapsin I which had been phosphorylated by Src in a buffer containing 220 mM glycine, 30 mM NaCl, 5 mM Tris-HCl, 4 mM HEPES (pH 7.4), 0.22 mM NaN3 and 100 µg/ml bovine serum albumin. After incubation, SV-bound synapsin I was separated by high-speed centrifugation (400,000 g for 30 minutes) through a 10% (w/v) sucrose cushion.

Aliquots of the resuspended pellets were subjected to SDS-PAGE followed by 32P radiography and visualized by autoradiography. In parallel, aliquots of the resuspended pellets were subjected to SDS-PAGE followed by immunoblotting with synapsin I-specific antibodies, to follow both the dephosphorylated and phosphorylated forms of synapsin I in the same samples. The recovery of SVs, used to correct the amounts of bound synapsin I, was determined by immunoblotting with anti-synaptophysin antibodies.

Actin-binding and actin-bundling assays

G-actin (5 µM) was polymerized for 1 hour at room temperature by the addition of 10 mM MgCl2 and incubated at 30°C for 2 hours with synapsin I (0.3 µM), which had been phosphorylated by Src as described above. The incubation was carried out in a buffer containing 12.5 mM NaCl, 0.6 mM ATP, 100 mM KCl, 1.2 mM MgCl2, 1.5 mM 2-mercaptoethanol, 6 mM HEPES, 8 mM MgCl2. After the incubation, samples were subjected to high-speed centrifugation (400,000 g for 30 minutes at 4°C) for recovery of total F-actin (Bährler and Greengard, 1987). The actin pellet and supernatant fractions were solubilized in SDS-sample buffer and subjected to SDS-PAGE, as described below. Actin recovery in the various fractions was determined by Coomasie blue staining of the gels, whereas the amount of synapsin I bound to actin filaments or free in the supernatant was determined by 32P-radioactivity counting and immunoblotting with synapsin-I-specific antibodies.

Crosslinking of synapsin I oligomers

The formation of synapsin I oligomers was assessed by crosslinking experiments using DSS. Briefly, 0.3 µM dephosphorylated synapsin I or synapsin I which had been previously phosphorylated by Src as described above was incubated for 60 minutes at 30°C in a buffer containing 20 mM HEPES, pH 7.4, 10 mM MnCl2, 2 mM EGTA, 0.4 mM Na3VO4, 10 mM magnesium acetate, 1 mM ATP, 5 mM MgCl2. Slices were incubated for 1 hour at 37°C in a 15% (w/v) dextran gels, transferred to nitrocellulose membranes and detected by immunoblotting with anti-synapsin-I and anti-Tyr-P-synapsin-I antibodies.

Rat cerebral cortex slices

Acute cortical slices (300 µm thick) were prepared from male Sprague-Dawley rats (150-200 g) with a McIlwain tissue chopper and incubated as described previously (Siciliano et al., 1994). Briefly, slices were dissociated in Ca2+-free artificial cerebrospinal fluid (ACSF) at 4°C and equilibrated at pH 7.4 with O2:CO2 (95:5 v/v). Slices were transferred to polypropylene tubes (three slices per tube) containing 2 ml Ca2+-free-ACSF at 37°C. After 10 minutes, the medium was aspirated and replaced by 1.8 ml ACSF containing 1.1 mM CaCl2 and 0.83 mM MgCl2. Slices were incubated for 1 hour at 37°C in a moderate agitation, under a humidified atmosphere of O2:CO2 (95:5 v/v) before depolymerization with 40 mM KCl (final concentration) for 2 minutes. Tetrodotoxin (1 µM) was added to the incubation medium to prevent indirect effects of neuronal firing and had no effect on synapsin I phosphorylation (Laura Perlini, F.B. and F.V., unpublished results). After treatment, slices were solubilized by sonication in 200 µl of a 100°C solution of 1% (w/v) SDS, 1 mM Na3VO4, added with Laemmli sample buffer and further boiled for 5 minutes.

DNA constructs

Green fluorescent-protein-tagged rat synapsin Ia (GFPSynapla) was kindly donated by Hung-Teh Kao (Brown University, Providence, RI). Cherry-tagged Synipla was generated by using the mammalian expression vector pmCherry-C1 (Clontech, Mountain View, CA) that encoded for the red fluorescent protein fused at the N-terminus of rat SynIa. The non-phosphorylatable SynIa mutant (Y301F-SynIa) was generated by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Milan, Italy) following the manufacturer’s procedure with two antiparallel primers carrying codons for the required substitution. Primers: forward, 5'GTCGCA CTG ACT AAG ACA TTT GCC ACT GCT GAG CCG TTC-3'; reverse, 5'-GAA GGG CTC AGC AGT GGC AAA TGT CTT AGT CAG TGC CAC-3'.

Cell culture and transfection

Hippocampal cells were prepared from Synl-KO mouse embryos as previously described (Baldelli et al., 2007) with minor modifications. Pregnant mice were injected with 100 ng progesterone on day 15.1 of gestation, and 18 day embryos were subjected to cesarean section. The isolated hippocampal neurons were plated onto ECM-coated coverslips (Sigma) at 600 cells/mm² and maintained in a culture medium consisting of Neurobasal, B-27 (1:50 v/v), glutamine (1% v/v), penicillin-streptomycin (1% v/v) (all from Invitrogen). A medium replacement was performed the day after plating and half-volume medium replacement was performed at 9-11 days in vitro (DIV). Hippocampal neurons were co-transfected at 9-11 DIV with the super ecliptic GFP-synaptophysin chimera construct (Syph; kind gift from Yongling Zhu and Charles F. Stevens, The Salk Institute, La Jolla, CA) together with Cherry-tagged WT- or Y301F-Synapla, using Lipofectamine 2000 (Invitrogen).

Live-cell imaging and quantification

Optical recordings were performed at 14-21 DIV (i.e. 5-12 days after transfection) on coverslips using a laminar-flow perfusion and stimulation chamber (volume
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