Phosphorylation by cAMP-dependent protein kinase is essential for synapsin-induced enhancement of neurotransmitter release in invertebrate neurons

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Summary

Synapsins are synaptic vesicle-associated phosphoproteins involved in the regulation of neurotransmitter release and synapse formation; they are substrates for multiple protein kinases that phosphorylate them on distinct sites. We have previously found that injection of synapsin into *Helix* snail neurons cultured under low-release conditions increases the efficiency of neurotransmitter release. In order to investigate the role of phosphorylation in this modulatory action of synapsins, we examined the substrate properties of the snail synapsin orthologue recently cloned in *Aplysia* (apSyn) for various protein kinases and compared the effects of the intracellular injection of wild-type apSyn with those of its phosphorylation site mutants. ApSyn was found to be an excellent in vitro substrate for cAMP-dependent protein kinase, which phosphorylated it at high stoichiometry on a single site (Ser-9) in the highly conserved domain A, unlike the other kinases reported to phosphorylate mammalian synapsins, which phosphorylated apSyn to a much lesser extent. The functional effect of apSyn phosphorylation by cAMP-dependent protein kinase on neurotransmitter release was studied by injecting wild-type or Ser-9 mutated apSyn into the soma of *Helix* serotonergic C1 neurons cultured under low-release conditions, i.e. in contact with the non-physiological target neuron C3. In this model of impaired neurotransmitter release, the injection of wild-type apSyn induced a significant enhancement of release. This enhancement was virtually absent after injection of the non-phosphorylatable mutant (Ser-9→Ala), but it was maintained after injection of the pseudophosphorylated mutant (Ser-9→Asp). These functional effects of apSyn injection were paralleled by marked ultrastructural changes in the C1 neuron, with the formation of extensive interdigitations of neurite-like processes containing an increased complement of C1 dense core vesicles at the sites of cell-to-cell contact. This structural rearrangement was virtually absent in mock-injected C1 neurons or after injection of the non-phosphorylatable apSyn mutant. These data indicate that phosphorylation of synapsin domain A is essential for the synapsin-induced enhancement of neurotransmitter release and suggest that endogenous kinases phosphorylating this domain play a central role in the regulation of the efficiency of the exocytotic machinery.

Key words: Exocytosis, Synaptic vesicles, Protein kinases, Cytoskeleton, Protein phosphorylation, Invertebrate neurons

Introduction

The synapsins are a family of synaptic vesicle (SV)-associated phosphoproteins involved in the regulation of neurotransmitter release (Greengard et al., 1993; Pieribone et al., 1995; Hilfiker et al., 1999; Chi et al., 2001) and synaptic plasticity (Rosahl et al., 1995; Humeau et al., 2001; Angers et al., 2002). Moreover, these proteins are implicated in neuronal development, regulating neurite outgrowth (Ferreira et al., 1994; Ferreira et al., 1995; Kao et al., 2002) and synaptogenesis (Ferreira and Rapoport, 2002). Increased synapsin levels promote the morphological and functional maturation of neurotransmitter release mechanisms in developing *Xenopus* neuromuscular synapses (Lu et al., 1992; Schaeffer et al., 1994; Valtorta et al., 1995). Similarly, synapsin overexpression in neuroblastoma-glioma cell lines induces the differentiation of presynaptic-like terminals and an increase in the number of releasable synaptic vesicles (Han et al., 1991; Sugiyama et al., 2000). Conversely, reduction of synapsin levels delays neuronal differentiation, axonal outgrowth and synaptogenesis (Ferreira et al., 1995; Ferreira et al., 2000; Chin et al., 1995).

The synapsins are composed of individual and shared domains (Hilfiker et al., 1999), some of which are highly conserved phylogenetically (Kao et al., 1999; Angers et al., 2002). They are major substrates for multiple protein kinases, including cAMP-dependent protein kinase (PKA), Ca^{2+}/calmodulin protein kinases (CaMK) I, II and IV, and mitogen-associated protein kinase/Erk 1/2 (MAPK/Erk). There are distinct phosphorylation sites on the synapsins (Greengard et

al., 1993; Jovanovic et al., 2000; Sakurada et al., 2002), but their presence varies in the different isoforms and orthologues.

Molluscan neurons offer unique advantages in studying the mechanisms regulating neurotransmitter release (Augustine et al., 1999; Smit et al., 2001; Kandel, 2001), including the possibility of injecting exogenous molecules directly into axonal or somatic giant presynaptic compartments (e.g. Fang et al., 1994; Hilfiker et al., 2001). The serotoninergic neuron C1 of the land snail *Helix pomatia* has been used to investigate the influence of the postsynaptic target on the efficiency of neurotransmitter release mechanisms in culture (Ghirardi et al., 2000; Ghirardi et al., 2001). We have recently found that the injection of mammalian synapsin I into C1 neurons cultured under low-release conditions (i.e. in contact with the non-physiological target neuron C3) increases the efficiency of the neurotransmitter release machinery (Fiumara et al., 2001). However, in those experiments it was not possible to assess the role of synapsin phosphorylation in enhancing neurotransmitter release, as the state of phosphorylation of the injected protein strongly influenced its diffusion along the neurites towards the neurotransmitter release sites (Fiumara et al., 2001).

In this study, we investigate the role of phosphorylation in the enhancing effect exerted by synapsin on neurotransmitter release from C1 neurons. To this aim, (1) we analyzed the substrate properties of a snail synapsin orthologue recently identified in *Aplysia* (apSyn) (Angers et al., 2002) for the protein kinases known to phosphorylate mammalian synapsins; (2) we developed C1-C3 co-cultures in soma-soma configuration, which allow the injection of proteins close to neurotransmitter release sites avoiding phosphorylation-dependent differences in axonal diffusion; and (3) we analyzed the effects of intracellular injection of wild-type apSyn and its phosphorylation site mutants on serotonin (5-hydroxy tryptamine, 5HT) release from C1 neuron somata. We found that phosphorylation of apSyn on a single residue in the NH_2-terminal domain A by PKA is essential for the synapsin-induced enhancement of neurotransmitter release and that this effect is accompanied by an ultrastructural rearrangement in the C1 neuron.

Materials and Methods

Materials

Leibovitz L15 culture medium (L15), protease type IX, bovine serum albumin (BSA), poly-L-lysine, Tris-HCl, saponin and Fast Green were from Sigma (Milano, Italy). Sylgard 184 silicone elastomer was from Dow Corning (Wiesbaden, Germany). Paraformaldehyde was from Merck (Milano, Italy). *Aplysia* hemolymph was collected from adult specimens of wild *Aplysia punctata* caught in the Adriatic sea. [γ-^{32}P] ATP (>3000 Ci/mmol), pGEX-2T, anti-glutathione S-transferase (GST) antibodies, glutathione-Sepharose 4B and highly purified thrombin were obtained from Amersham Pharmacia (Milano, Italy). Synapsin I was purified from bovine brain by detergent extraction as previously described (Bähler and Greengard, 1987) and stored at –80°C. The purified bovine heart catalytic subunit of PKA was from Sigma (Milano, Italy). Purified CaMK II and protein kinase C (PKC) were from Calbiochem (La Jolla, CA). Recombinant murine MAPK/Erk2 was from Upstate Biotechnology (Lake Placid, NY). Calmodulin was from Roche Biochemicals (Milano, Italy). Protein kinase activity was assessed using bovine synapsin I as substrate. Antibodies against mammalian and *Aplysia* synapsin were raised in our laboratory (Cibelli et al., 1996; Onofri et al., 1997). The pGEX-2T plasmid containing the cDNA of apSyn (clone 11.1) was a kind gift of Annie Angers and John H. Byrne (The University of Texas, Houston, TX). The site-directed mutagenesis kit was from Stratagene (La Jolla, CA). All other chemicals were of analytical grade.

Expression and purification of recombinant proteins

Bacterial cells (BL21 DE3 strain) were transformed to ampicillin resistance by electroporation with constructs containing pGEX-4T1 alone or pGEX-4T1 in frame with sequences encoding apSyn. Large-scale cultures of Luria broth containing ampicillin (100 μg/ml) were inoculated with small overnight cultures, grown at 37°C to log phase and induced with isopropyl β-D-thiogalactopyranoside (100 μM) for 3-5 hours. GST and GST/apSyn fusion proteins were extracted from bacterial lysates, purified to homogeneity by affinity chromatography on glutathione-Sepharose and dialyzed against 25 mM Tris-HCl, 50 mM NaCl, pH 7.4 (McPherson et al., 1994). For some experiments, the purified apSyn/GST fusion protein was subjected to cleavage with thrombin (1 U/mg fusion protein) for 1 hour at 22°C and repurified by a further passage through the glutathione-Sepharose column following the manufacturer's procedure.

Phosphorylation of *Aplysia* synapsin

For the analysis of synapsin phosphorylation by the catalytic subunit of PKA, bovine synapsin I (5 pmol) and apSyn 11.1 (50 pmol) were incubated in a buffer containing 50 mM HEPES, 10 mM $MgCl_2$, 1 mM ethyleneglycotetraacetic acid (EGTA), 0.1 mM β-mercaptoethanol, 50 mM Tris (pH 7.4) in the presence of 5 μg/ml of catalytic subunit of PKA. Phosphorylation of synapsins by activated MAPK/Erk2 was analyzed by incubation in a buffer containing 20 mM MOPS (pH 7.4), 20 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol, 30 mM β-glycerophosphate, 1.0 μg/ml purified active MAPK/Erk2. Phosphorylation of synapsins by CaMK II was assessed by incubation in buffer containing 50 mM HEPES (pH 7.4), 120 mM NaCl, 20 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.07 mM EGTA, 1 mM β-mercaptoethanol, 1 μM calmodulin and 0.6 μg/ml purified CaMK II. Finally, phosphorylation by PKC was analyzed by incubation in buffer containing 10 mM HEPES (pH 7.4), 15 mM KCl, 2 mM $MgCl_2$, 0.5 mM dithiothreitol and 1.0 μg/ml purified PKC. Protease inhibitors (0.2 mM PMSF, 2 μg/ml leupeptin and 10 μg/ml pepstatin) were added to all phosphorylation mixtures. After preincubation of the samples for 1 minute at 30°C, the incubations were started by the addition of 50 μM [γ-^{32}P] ATP (1-2 μCi/sample) and terminated at various times by the addition of concentrated sample buffer (Laemmli, 1970) and boiling for 2 minutes. Phosphorylated samples were resolved by SDS-PAGE on 10% polyacrylamide gels. After the run, gels were fixed, stained with Coomassie brilliant blue and exposed to Kodak X-Omat films. The amount of radioactivity incorporated into the synapsin bands was assessed by liquid scintillation counting of the radioactive bands excised from the gels.

Generation of *Aplysia* synapsin phosphorylation site mutants

In order to generate mutations in the apSyn region encompassing the putative PKA phosphorylation site (Ser-9 in apSyn 11.1), the QuickChange site-directed mutagenesis kit (Stratagene) was used following the manufacturer's procedure. The cDNAs containing the Ser-9→Ala (non-phosphorylatable mutant) and Ser-9→Asp (pseudophosphorylated mutant) were amplified by PCR using the following primers: Ser→Ala forward, 5′-CTACCTGCGCCGC-CGCTTCGCCTCGGGAGATCTC-3′; Ser→Ala reverse, 5′-GA-GATCTCCCGAGGCGAAGCGGCGGCGCAGGTAG-3′; Ser→Asp forward, 5′-CTACCTGCGCCGCCGCTTCGACTCGGGAGATCTC-3′; Ser→Asp reverse, 5′-GAGATCTCCCGAGTCGAAGCGGCG-GCGCAGGTAG-3′ and the amplified products were subcloned into pGEX-4T1 using standard recombinant DNA techniques (Sambrook

et al., 1989). The resulting expression plasmids were verified by sequencing and transformed into *E. coli* BL21(DE3). The resulting mutant proteins were purified as described above and subjected to PKA phosphorylation assays as described above.

Cell culture

Juvenile *Helix pomatia* snails (1-3 g) were purchased from local breeders. Cell cultures of *Helix* neuron somata were obtained using previous protocols for *Helix* (Ghirardi et al., 1996) and *Aplysia* (Klein, 1994; Manseau et al., 2001) neurons. Briefly, snails were anesthetized by the injection of isotonic $MgCl_2$ and their cerebral and buccal ganglia were isolated and digested for 3-4 hours at 34°C in 10 mg/ml protease type IX (Sigma) in L15 culture medium modified for *Helix* (Ghirardi et al., 1996). After digestion, ganglia were repeatedly washed in L15, pinned on a Sylgard-coated plastic dish in L15 and the connective sheaths covering the neurons were removed by fine forceps and scissors. Neurons C1, C3 and B2 (Cottrell, 1977; Prescott et al., 1997) were removed from the ganglia with their initial axonal segment and transferred to plastic culture dishes (Falcon 1008) containing 5% *Aplysia* hemolymph and 0.01% bovine serum albumin (BSA) in modified L15. Under these conditions, neurons failed to adhere to the plastic substrate and, after 12-48 hours, retracted their neurites, forming spherical somata devoid of processes. The floating somata were gently manipulated to form soma-soma pairs or left isolated according to the specific experimental requirements.

Electrophysiology

Conventional electrophysiological techniques were used for intracellular recordings (Ghirardi et al., 1996; Ghirardi et al., 2001). Signals were digitally recorded through a Digidata 1322A interface (Axon Instruments) and analyzed with Axoscope and Clampfit software packages (Axon Instruments).

Intracellular injections

In synapsin injection experiments, C1 neurons were paired with C3 neurons and loaded soon after with wild-type or mutated apSyn (11.1 isoform) (Angers et al., 2002). Neurons were impaled with a beveled glass electrode with the tip filled with the synapsin-containing buffer solution (protein concentration: 1.5 mg/ml) with 10% (v/v) of a saturated Fast Green solution (Humeau et al., 2001), and loaded with short pressure pulses (10-20 pulses of 0.3-0.5 seconds; 2-20 psi) delivered through a pneumatic picopump (PV820, WPI) connected to the electrode holder. The injection procedure was monitored under visual and electrophysiological control and stopped when the cell soma was uniformly green. Cells with morphological abnormalities or with alterations in resting potential and input resistance after the injection were discarded.

Evaluation of neurotransmitter release

The neurosecretory capabilities of the serotonergic C1 neuron somata (Osborne, 1977), cultured in isolation or paired with C3 neurons, were tested by using a 5HT-sensitive B2 neuron soma as an assay (sniffer) cell (Altrup and Speckmann, 1994; Ghirardi et al., 2001). The sniffer cells were transferred from the non-adhesive culture dish to a poly-L-lysine-coated recording dish, pre-treated with *Aplysia* hemolymph, which allows the micromanipulation of cells for some minutes before their adhesion to poly-L-lysine (Ghirardi et al., 1996), and containing L15 medium. After impalement with intracellular electrodes, the membrane potential of the B2 sniffer neuron was kept at –80 mV and the C1 and B2 somata were micromanipulated in contact with each other. Sniffer cells with unstable membrane potential or with a resting potential below –40 mV were discarded. Within a few seconds of contact, the C1 neuron was intracellularly stimulated with depolarizing current pulses to fire a train of action potentials (10 Hz for 10 seconds) while the depolarization of the sniffer neuron, induced by 5HT released from the C1 soma, was recorded. The amplitude of the sniffer depolarization was measured at its peak value after the end of C1 firing with respect to the baseline value prior to C1 stimulation.

Cell imaging

Phase-contrast video micrographs of cultured cells were digitally acquired from an Eclipse TE200 inverted microscope (Nikon, Tokyo, Japan) with a 20× objective through a Monochrome QE camera (Mediacybernetics, Silver Spring, MD) and processed using Photoshop 6.0 software (Adobe Systems, San Jose, CA).

Electron microscopy

Electrophysiologically tested cell pairs were fixed for 30 minutes at room temperature with 2% formaldehyde (freshly prepared from paraformaldehyde), 1% glutaraldehyde, 1% sucrose in 100 mM cacodylate buffer (pH 7.4), washed five times for 30 minutes in 100 mM cacodylate buffer (pH 7.4), and post-fixed for 1 hour at room temperature with 1% OsO_4 in the same buffer. The cells were subsequently washed five times for 5 minutes with 100 mM cacodylate buffer, dehydrated and embedded in Epon 812 as described (Ceccarelli et al., 1973). Silver-gray sections were cut on an Ultracut microtome (Reichert-Jung, Wien, Austria), triple-stained with 4% uranyl acetate before and after 0.4% lead citrate, and examined in a Hitachi H-7000 electron microscope (Hitachi, Tokyo, Japan).

Statistical analysis

All values are expressed as mean±s.e.m. Time-courses of phosphorylation were fitted by using the Sigmaplot 8.0 (SPSS Inc.) non-linear fitting procedures. Statistical significance was assessed by Student's *t*-test or one-way analysis of variance (ANOVA) followed by the Newman-Keuls post hoc test. In all instances a value of $P<0.05$ was considered as statistically different.

Results

ApSyn is phosphorylated by multiple protein kinases and is a preferential substrate for PKA in the A domain

Analysis of the primary structure of the *Aplysia* synapsin orthologue (apSyn 11.1) (Angers et al., 2002) revealed the high phylogenetic conservation of the PKA/CaMKI/IV phosphorylation site located in the N-terminal A domain (Ser-9) and the presence of additional consensus sequences for phosphorylation by MAPK/Erk (Ser-36, Ser-42), PKC (Ser-64, Ser-138, Thr-220, Ser-281) and possibly CaMK II (Ser-58, Ser-389) that are not conserved with respect to mammalian synapsins. In addition, it has been recently reported that apSyn is phosphorylated in intact *Aplysia* ganglia by treatment with transforming growth factor (TGF)-β1 and 5HT through activation of the PKA and MAPK pathways (Chin et al., 2002; Angers et al., 2002). Thus, we analyzed the in vitro substrate properties of apSyn for various protein kinases known to phosphorylate mammalian synapsins. In order to avoid interference by GST in the phosphorylation reactions, purified recombinant apSyn/GST fusion protein was cleaved with thrombin and repurified before incubation for various times with purified PKA, MAPK/Erk, PKC and CaMK II (Fig. 1, Fig. 2A).

Addition of purified catalytic subunit of PKA induced a rapid and marked phosphate incorporation into purified recombinant

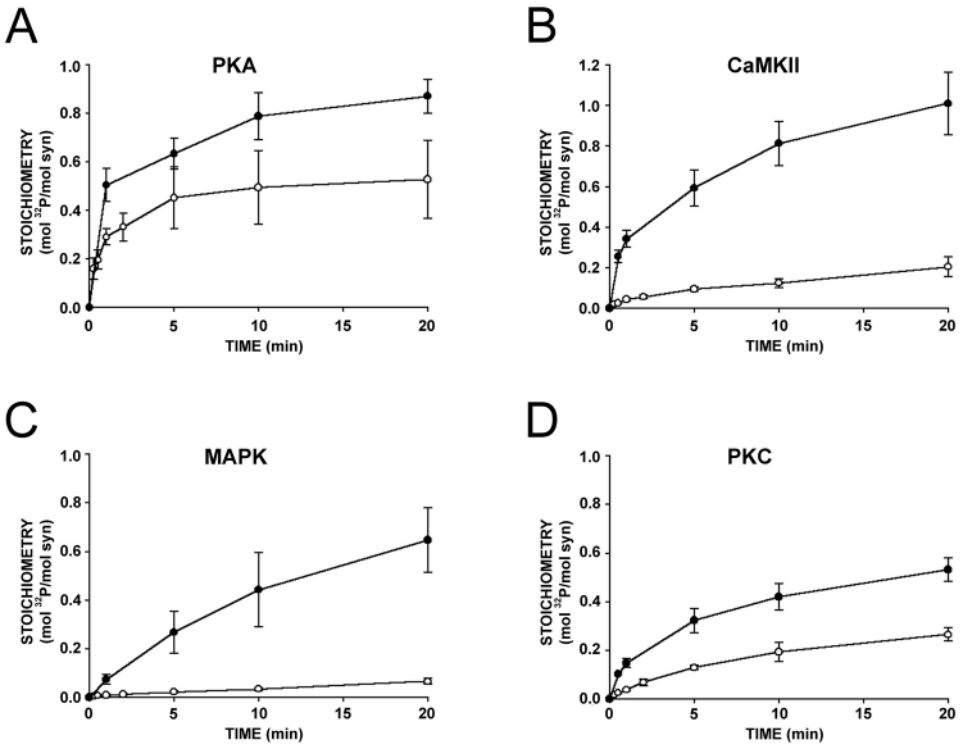

Fig. 1. Time-course and stoichiometry of apSyn phosphorylation by distinct protein kinases. Purified bovine synapsin I (5 pmol; closed symbols) or recombinant apSyn (clone 11.1; 50 pmol; open symbols) cleaved from GST and repurified was incubated at 30°C for the indicated time periods with either the catalytic subunit of PKA (A), CaMK II (B), MAPK/Erk2 (C) or PKC (D) in the presence of radioactive ATP. Phosphorylation stoichiometries (mol [^{32}P]/mol synapsin), calculated at various incubation times, are shown as mean±s.e.m. (*n*=4-5).

apSyn, with a time-course similar to that of native mammalian synapsin I (τ=0.86±0.16 and 1.02±0.37 minutes for apSyn and mammalian synapsin I, respectively) and a stoichiometry of 0.52±0.16 mol phosphate/mol apSyn after 30 minutes of incubation (Fig. 1A, Fig. 2A). ApSyn could also be phosphorylated by the other kinases tested, but to a much lesser extent. Incubation with either activated recombinant MAPK/Erk or purified CaMK II in the presence of Ca^{2+} and calmodulin promoted incorporation of radioactive phosphate in both apSyn and bovine synapsin I, but the phosphorylation stoichiometry was much lower in the snail orthologue than in the mammalian protein (Fig. 1B,C). Relatively low stoichiometric ratios were obtained after phosphorylation with PKC for both apSyn and bovine synapsin I, confirming that the synapsins are not preferential substrates for this kinase (Fig. 1D).

As apSyn was an excellent substrate for PKA, but not for the other kinases, we further investigated this phosphorylation in order to identify the phosphorylation site(s) and address the physiological importance of PKA-mediated phosphorylation for the effects of apSyn on neurotransmitter release. As mentioned above, a highly conserved consensus sequence for PKA encompassing Ser-9 is present in the NH_2-terminal domain A of apSyn. Thus, using site-directed mutagenesis, we mutated the putative phosphorylation site by substituting alanine for serine-9 to generate a non-phosphorylatable mutant (apSynALA9), and aspartate for serine-9 to mimic a persistent phosphorylation of the protein (pseudophosphorylated mutant; apSynASP9). Subsequent phosphorylation of wild-type apSyn and its phosphorylation site mutants with PKA revealed that both mutations completely prevented phosphate incorporation, indicating that Ser-9 in domain A is the only apSyn site that is phosphorylated by PKA (Fig. 2B).

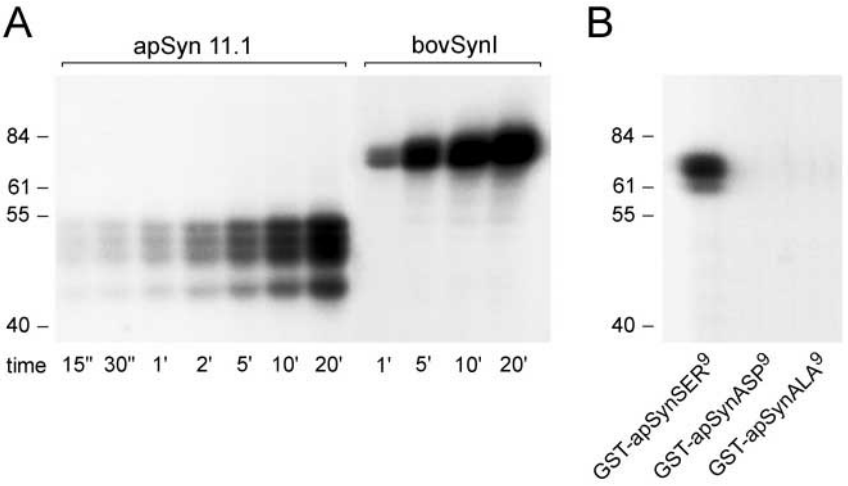

Fig. 2. PKA phosphorylates apSyn at a single site in domain A. (A) Radioactive phosphate incorporation into recombinant apSyn 11.1 or bovine synapsin I (5 pmol) as a function of the incubation time demonstrates that the snail synapsin orthologue is an excellent substrate for PKA. The multiple bands observed in the apSyn lanes are probably caused by partial cleavage by thrombin. (B) Mutation of Ser-9 in the conserved PKA consensus sequence present in apSyn domain A into alanine (apSynALA9) or aspartate (apSynASP9) completely abolishes [^{32}P] incorporation after incubation with the PKA catalytic subunit for 20 minutes, indicating that Ser-9 is the only phosphorylation site for PKA. The positions of molecular mass standards are shown on the left in kDa.

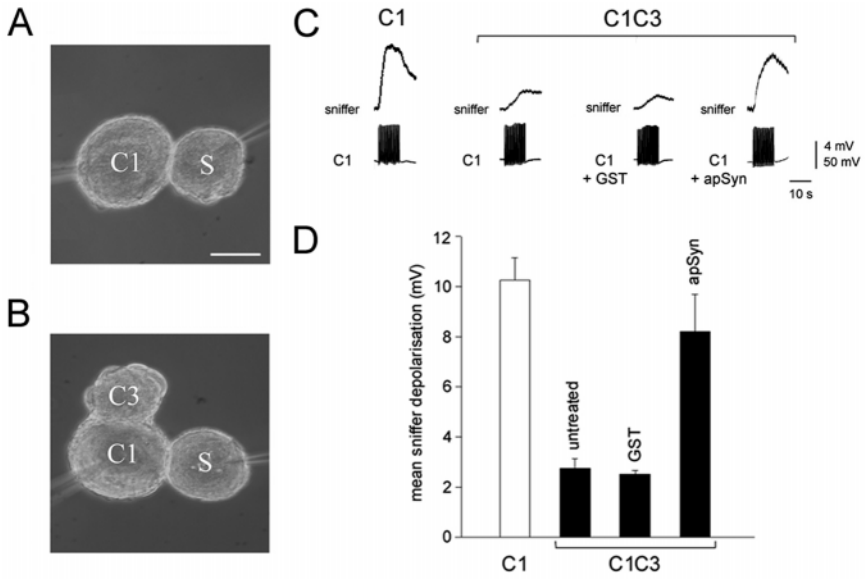

Fig. 3. Intracellular injection of apSyn enhances evoked neurotransmitter release from C1 neuron somata cultured under low-release conditions. (A) Phase-contrast image of the soma of a C1 neuron immediately after contact with a 5HT-sensitive sniffer cell (S). Bar, 50 μm. (B) Phase-contrast image of a C1-C3 soma-soma co-culture. Twenty-four hours after C1-C3 pairing, a sniffer cell (S) was micromanipulated to contact the membrane of the C1 soma to detect neurotransmitter release. Calibration as in A. (C) Sample intracellular recordings of the sniffer cell depolarization (upper trace) induced by a train of action potentials (10 Hz for 10 seconds) in the C1 soma (lower trace) under different experimental conditions. The basal membrane potential of the sniffer cell was kept at –80 mV. (D) The mean sniffer depolarization measured after stimulation of C3-paired C1 somata is significantly lower than the mean depolarization induced by stimulation of isolated C1. The amplitude of sniffer depolarization is significantly higher 24 hours after injection of the GST-apSyn fusion protein. No change is induced by injection of GST alone.

ApSyn enhances evoked neurotransmitter release from the soma of C1 neurons cultured under low-release conditions

The soma of neurons of various invertebrate species is capable of releasing neurotransmitter in the absence of neurite outgrowth (e.g. Haydon, 1988; Lovell et al., 2002). We tested whether the soma of *Helix* C1 neurons in culture is also capable of evoked neurotransmitter release. As B2 neurons are postsynaptic targets of the serotonergic C1 neurons in vivo (Cottrell, 1977), we used a 5HT-sensitive B2 soma, micromanipulated in contact with C1, as a detector (‘sniffer’) of 5HT release (Ghirardi et al., 2001) (Fig. 3A). The intracellular stimulation of the isolated C1 neuron to fire action potentials at 10 Hz for 10 seconds led to a slow and long-lasting depolarization of the B2 sniffer resulting from the evoked 5HT release (mean peak amplitude: 10.26±0.89 mV, n=8) (Fig. 3C,D). The amplitude of the sniffer depolarization remained substantially stable in repeated trials of C1 stimulation with the same protocol, for at least 30 minutes after the first test (not shown), suggesting the presence of an efficient machinery for evoked neurotransmitter release in the isolated C1 soma.

In previous experiments in which the C1 neuron was co-cultured in contact with a non-physiological target (e.g. the motoneuron C3) in neurite-neurite configuration, we observed a reduced number of 5HT-containing varicosities and an impaired capability of neurotransmitter release with respect to C1 neurons grown in isolation (Ghirardi et al., 2000). We investigated whether the downregulation of neurotransmitter release induced by contact with the non-physiological target was also present in the soma-soma culture configuration. Twenty-four hours after C1-C3 soma-soma pairing (Fig. 3B), the mean amplitude of the sniffer depolarization induced by the stimulation of C1 somata co-cultured in contact with the C3 soma was significantly lower (2.74±0.38 mV, n=17; P<0.01, Newman-Keuls post hoc test compared to isolated C1) than that measured after stimulation of control C1 somata cultured in isolation (Fig. 3C,D). These results show that the low-release condition of the C1 neuron after contact with the non-physiological target C3 was also maintained in the soma-soma configuration.

The C1-C3 neurite-neurite co-culture was previously used as a model system to investigate the role of exogenous synapsin in modulating serotonin release from C1 neuritic terminals (Fiumara et al., 2001). Thus, we tested whether the synapsin-induced modulation of neurotransmitter release was also present in soma-soma C1-C3 co-cultures injected with the snail synapsin orthologue apSyn. C3-paired C1 somata were intracellularly pressure-injected with an apSyn/GST fusion protein, whereas control experiments were injected with either vehicle or GST alone. Twenty-four hours after injection, the release of neurotransmitter from C1 somata was tested by using a sniffer neuron brought in contact with the C1 membrane (Fig. 3C). The mean amplitude of the sniffer depolarization induced by firing (10 Hz for 10 seconds) of apSyn-injected C1 neurons was significantly higher (8.20±1.49 mV, n=15) than that observed after firing of uninjected (see above) or GST-injected C1 neurons (2.53±0.22 mV, n=9; P<0.01, Newman-Keuls post hoc test) (Fig. 3D). The persistence of apSyn/GST fusion protein in C1 neurons 24 hours after injection was verified by immunocytochemistry with an anti-GST antibody (data not shown).

Phosphorylation of apSyn domain A is necessary for the enhancement of neurotransmitter release

To investigate whether phosphorylation of the domain A of apSyn is involved in the synapsin-induced enhancement of neurotransmitter release from the C1 neuron, we compared the effects of the intracellular injection of wild-type apSyn with those of its non-phosphorylatable (apSynALA9) or pseudophosphorylated (apSynASP9) mutants expressed as fusion proteins with GST. C3-paired C1 somata were injected with apSyn, apSynASP9, apSynALA9 or GST alone, and tested for 5HT release 24 hours later. Representative recordings are shown in Fig. 4A. The mean sniffer depolarization amplitude recorded after stimulation of C1 neurons loaded with

apSyn and apSynASP9 (7.69±2.24 mV, *n*=10; and 7.26±1.80 mV, *n*=11, respectively) was significantly higher than that recorded after the stimulation of C1 neurons injected with GST (2.50±0.16 mV, *n*=16; *P*<0.02, Newman-Keuls post hoc test). On the contrary, the mean sniffer depolarization amplitude measured after stimulation of C1 neurons loaded with apSynALA9 was not significantly different from that recorded after firing of GST-injected neurons (3.89±0.52 mV, *n*=15; *P*=0.42) (Fig. 4B). These results indicate that the endogenous phosphorylation of the A domain of wild-type apSyn by either PKA or CaMK I/IV is necessary for the enhancement of neurotransmitter release, as the effect is retained by the pseudophosphorylated form and virtually lost with the non-phosphorylatable mutant.

The functional effects of apSyn are associated with a phosphorylation-dependent ultrastructural rearrangement of C1 neurons

Increased synapsin levels are able to induce morphological changes in the ultrastructure of both neuronal and non-neuronal cell types, including cytoskeletal remodeling and SV reorganization (Han et al., 1991; Han and Greengard, 1994; Valtorta et al., 1995). To investigate the possible ultrastructural correlates of the functional effects induced by synapsin injection in C1 neurons, we fixed and processed for electron microscopy C1-C3 pairs in which the C1 neuron had been loaded with GST, apSyn or its non-phosphorylatable mutant apSynALA9. The cells were fixed 24 hours after injection, soon after being electrophysiologically tested. We did not analyze the ultrastructure of cells injected with apSynASP because this mutant protein had functional effects virtually indistinguishable from those of apSyn. We found that the most prominent ultrastructural differences between the experimental groups could be observed in the region of C1 neurons contacting the non-physiological partner C3 (Fig. 5A-D). In control C1-C3 pairs (i.e. after GST injection in C1), the two cells made contact with each other only by means of few embricated cellular processes (Fig. 5A,A′). Virtually no SV clustering or synaptic specializations could be observed in these samples, in contrast to that usually observed in synaptically connected pairs of molluscan neuron somata (Ohnuma et al., 2001; Klein, 1994). The SV profiles present in these processes were identified as belonging to either the C1 or the C3 neuron because of their morphological features (Pentreath, 1976; Elekes and Ude, 1993). As described for C1 neurons in intact ganglia (Pentreath and Cottrell, 1974; Pentreath, 1976), cultured C1 neurons contained large dense core SVs in variable association with small clear SVs (Fig. 5E), whereas C3 neurons contained dense and slightly elongated granules (Fig. 5F), occasionally clustered in the soma and scarcely present, as isolated elements, in the area of contact with C1.

The injection of C1 neurons with wild-type apSyn was followed by the formation of dense interdigitations of microtubule-packed neurite-like processes, leading to a several-fold thickening of the area occupied by their meshwork (mean thickness: 16.15±3.46 μm, *n*=3) with respect to GST-injected control pairs (mean thickness: 4.02±1.44 μm, *n*=3; *P*<0.02, Newman-Keuls post hoc test) (Fig. 5B′,D). The enlargement of the area of process interdigitation induced by

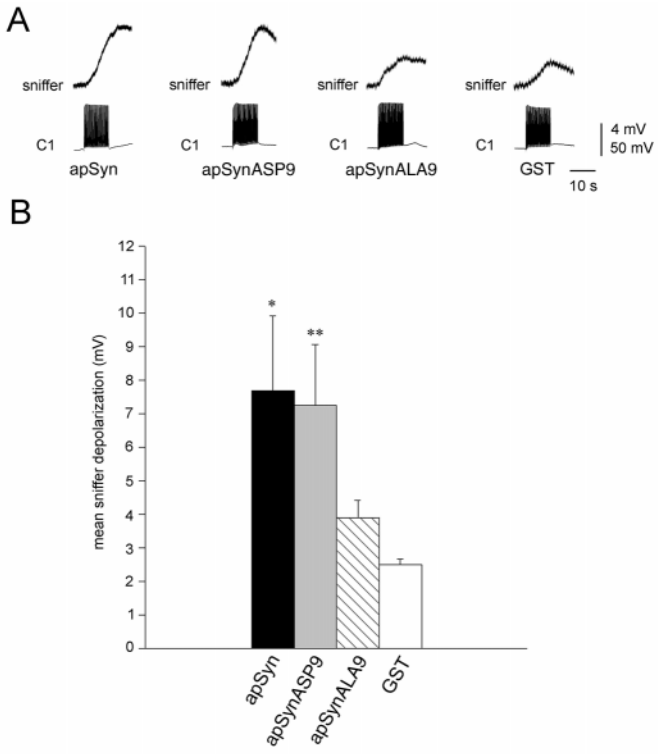

Fig. 4. The apSyn-induced enhancement of neurotransmitter release requires phosphorylation of domain A. (A) Sample recordings of sniffer depolarization induced by C1 firing (10 Hz for 10 seconds) representative of the different experimental groups in which the soma of the C1 neuron paired with C3 was loaded with either wild-type apSyn, apSynASP9, apSynALA9 or GST. (B) Bar graph showing that the mean amplitude of sniffer depolarization recorded after firing of C1 neurons loaded with either apSyn or the pseudophosphorylated apSynASP9 mutant was significantly higher than that recorded after the stimulation of control neurons injected with GST alone. In contrast, the mean sniffer depolarization amplitude measured after stimulation of C1 neurons loaded with the non-phosphorylatable apSynALA9 mutant was not different from that recorded after firing of GST-injected neurons.

wild-type apSyn injection was associated with the appearance of numerous clusters of dense core SVs typical of the C1 neuron (Fig. 5B) that were virtually absent in control C1 neurons. Moreover, in apSyn-injected C1 neurons, numerous processes filled with SV clusters were found to grow on the external borders of the thickened interdigitation area and, in some cases, to sprout on the free surface of the C1 neuron, where the sniffer cells were positioned to detect neurotransmitter release (Fig. 5B″). These processes were absent under control conditions (Fig. 5A″). The ultrastructural changes observed after apSyn injection were virtually absent when the C1 neuron was injected with the non-phosphorylatable mutant apSynALA9. Under the latter condition, the contact area between the C1 and C3 neurons was not significantly different from that observed after the injection of GST (mean thickness: 3.32±0.86 μm, *n*=3; *P*=0.45); only a thin layer of cellular processes was visible between the two cells, with the presence of scarce and disperse SV profiles (Fig. 5C-C″,D).

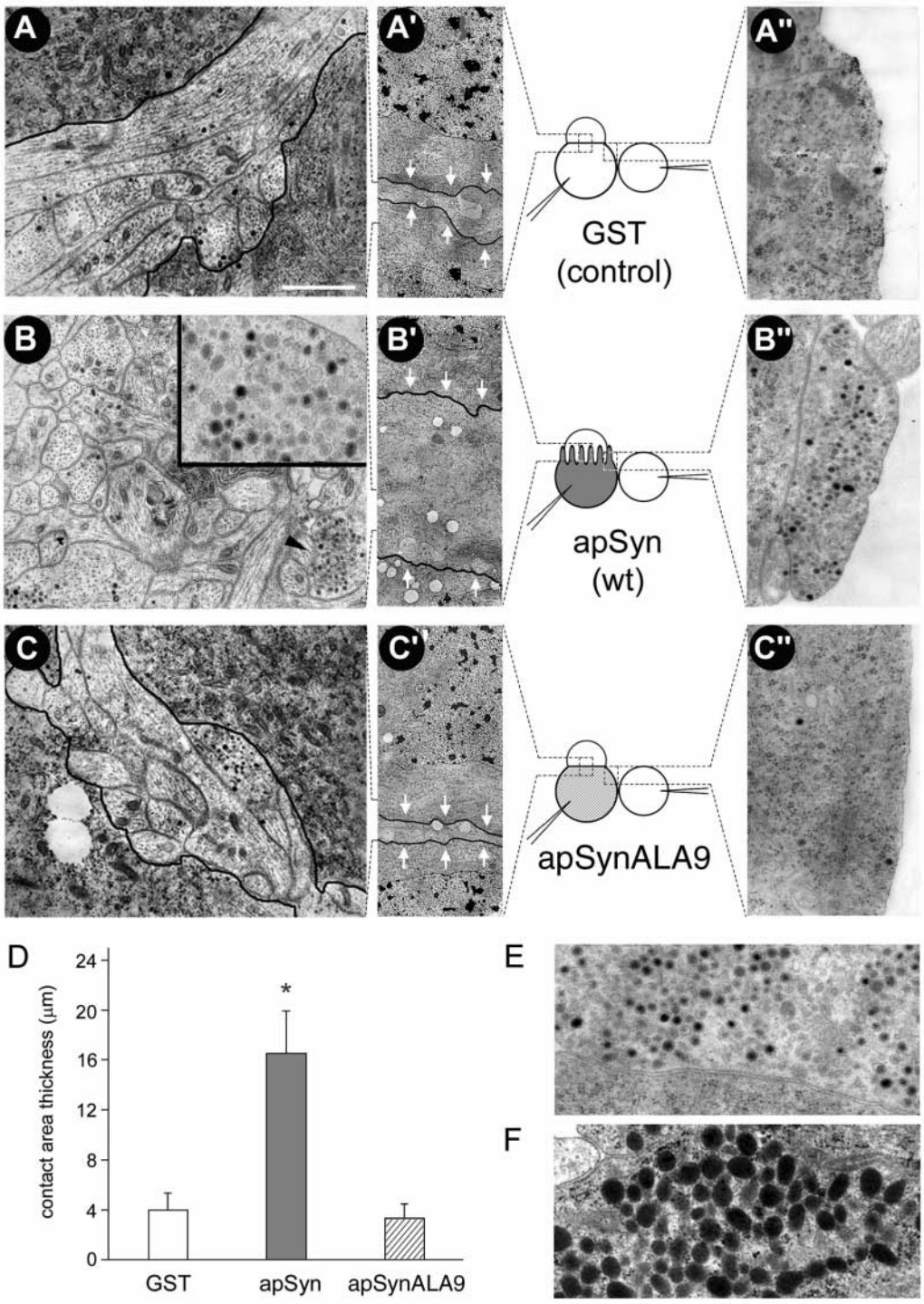

Fig. 5. ApSyn induces ultrastructural changes in C1 neurons in a phosphorylation-dependent manner. (A-C) Fine structure of contact areas between C1 and C3 neurons of soma-soma pairs in which C1 has been injected with GST (A-A″), apSyn (B-B″) or apSynALA9 (C-C″). The boundaries of the meshwork of interdigitating cellular processes between C1 and C3 somata are highlighted in black (arrows). Note the marked increase in the thickness of this area in the sample injected with apSyn (B-B′) with respect to either GST- or apSynALA9-loaded samples (A-A′ and C-C′, respectively). In the apSyn-injected samples the area of contact was characterized by a dense meshwork of neurite-like processes filled with microtubules and clusters of dense core SV profiles typical of the C1 neuron (see arrowhead in B and high magnification inset). Clusters of SVs were also visible in processes extending outside the contact area (B″). These processes were virtually absent in C1-C3 pairs loaded with GST or apSynALA9 (A-A″ and C-C″). (D) Bar graph showing the differences in the thickness of the meshwork of embricated processes between C1 and C3 in the three experimental groups. When the C1 neuron was loaded with apSyn, the interdigitation area was significantly thicker than in control conditions (GST injection) or after injection of the non-phosphorylatable mutant apSynALA9. (E) A cluster of dense core SVs typical of the C1 neuron. (F) Large neurosecretory granules observed in the soma of C3. Bar, 1.8 μm for panels A-C; 8 μm for panels A′-C′; 1 μm for panels A″-C″; 0.5 μm for panels E,F and inset in panel B.

Discussion

In various experimental systems, synapsins have been shown to play important roles in regulating neuronal development and differentiation, neurite outgrowth, synaptogenesis and neurotransmitter release (Hilfiker et al., 1999; Ferreira and Rapoport, 2002). Here, we used the somata of cultured molluscan neurons as a useful model to study the modulatory effects of invertebrate synapsins and of their phosphorylation in the regulation of neurotransmitter release.

The *Helix* serotonergic C1 neurons cultured in the absence of neuritic processes are capable of releasing neurotransmitter in response to electrical stimulation, indicating that the basic exocytotic machinery is present in the isolated soma prior to contact with postsynaptic partners. This exocytotic ability can be partially depressed by contact with a non-physiological target neuron, similar to that observed in neurite-neurite co-cultures (Ghirardi et al., 2000). In the latter low-release conditions, the intracellular injection of mammalian synapsin I into C1 neurons was able to enhance their neurotransmitter release capability (Fiumara et al., 2001). Here, we have found that a similar effect was exerted after injection of a molluscan synapsin orthologue in C1 neurons cultured under the same low-release conditions (C1-C3 co-culture) in the absence of neurite outgrowth (i.e. soma-soma configuration), as evaluated 24 hours after the injection. The enhancement of evoked 5HT release was paralleled by a significant increase in the extension of neurite-like SV-enriched processes extended by the C1 neuron in the contact area with the non-synaptic partner neuron C3.

To investigate the role of phosphorylation in the synapsin-induced modulation of C1 neurotransmitter release, we first characterized in detail the substrate properties of apSyn in vitro. ApSyn contains consensus sequences for several protein kinases and is phosphorylated in intact *Aplysia* ganglia by PKA and TGFβ1-stimulated MAPK/Erk (Chin et al., 2002; Angers et al., 2002). We found that this snail synapsin orthologue is an excellent substrate for PKA, which phosphorylates it stoichiometrically at a single site in the highly conserved A domain, whereas its phosphorylation by MAPK/Erk, CaMK II and PKC occurs to a much lower extent. In neurons, the cAMP/PKA pathway is involved in a plethora of functions, including neuronal differentiation, neurite outgrowth, synaptogenesis and neurotransmitter release (Song and Poo, 1999; Yao et al., 2000; Hilfiker et al., 2001; Kao et al., 2002). The most abundant neuronal substrates for PKA are the synapsins, whose PKA phosphorylation site in domain A is highly conserved in the various isoforms and across species, with high sequence identity among mammalian synapsins and other vertebrate and invertebrate synapsin orthologues (Kao et al., 1999). It is noteworthy that the very recently cloned *Helix* synapsin orthologue (C.M. and P.G.M., unpublished) is highly homologous to apSyn with maximal sequence conservation in the NH_2-terminal domain A containing the PKA phosphorylation site (77% sequence identity).

The finding that apSyn is a preferential substrate for phosphorylation by PKA at a single site in the A domain led us to test the hypothesis as to whether phosphorylation of this site might be relevant in the enhancing effect exerted by apSyn on the C1 neurosecretory capability. Interestingly, we found that the increase in neurotransmitter release and in the development of SV-enriched processes in C1 neurons observed after the injection of wild-type apSyn were virtually absent after the injection of an apSyn mutant bearing a Ser→Ala point mutation in the PKA phosphorylation site. On the other hand, injection of a pseudophosphorylated apSyn mutant bearing a negative charge (mimicking permanent phosphorylation of the PKA site) induced an increase in neurotransmitter release that was indistinguishable from that induced by wild-type apSyn. These data strongly indicate that the form of synapsin responsible for the enhancement of the efficiency of the C1 exocytotic machinery is that phosphorylated at Ser-9 in the NH_2-terminal A domain.

The molecular mechanism(s) by which domain A phosphorylation brings about the functional and structural changes in low-releasing C1 neurons is not known. In neurons, synapsins are transported down the axon and concentrate in growth cones and presynaptic boutons, where they participate in the assembly and maintenance of SV pools and in a rearrangement of the presynaptic actin cytoskeleton. This action probably results from the ability of synapsin to bind SVs, actin filaments and microtubules, to mediate SV clustering and to promote polymerization of actin monomers (Baines and Bennett, 1986; Bahler and Greengard, 1987; Benfenati et al., 1992; Valtorta et al., 1992; Benfenati et al., 1993; Ceccaldi et al., 1995).

Biochemical studies have shown that phosphorylation of synapsin at the A domain site can modulate the interactions with actin (Benfenati et al., 1992; Valtorta et al., 1992; Nielander et al., 1997) and SVs (Hosaka et al., 1999), possibly altering the aggregation of SVs and the general cytoskeletal architecture of the presynaptic terminal. The same site is also phosphorylated by CaMK I and IV, which have been suggested to play a role in the activity-dependent modulation of presynaptic function, by modulating SV mobilization and turnover in hippocampal cell cultures (Chi et al., 2001; Chi et al., 2003). Moreover, it has recently been shown that PKA-dependent synapsin phosphorylation regulates neurite outgrowth (Kao et al., 2002) and is involved in short-term plasticity of the sensorimotor synapse of *Aplysia* (Angers et al., 2002).

The present study highlights the key role of phosphorylation of synapsin domain A in the modulation of the exocytotic machinery and strongly suggests that the activation of PKA and/or of other kinases phosphorylating the same synapsin site plays a central role in the regulation of neurotransmitter release and, ultimately, of synaptic strength.

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