PKA-catalyzed phosphorylation of tomosyn and its implication in Ca\(^{2+}\)-dependent exocytosis of neurotransmitter

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eurotransmitter is released from nerve terminals by Ca\(^{2+}\)-dependent exocytosis through many steps. SNARE proteins are key components at the priming and fusion steps, and the priming step is modulated by cAMP-dependent protein kinase (PKA), which causes synaptic plasticity. We show that the SNARE regulatory protein tomosyn is directly phosphorylated by PKA, which reduces its interaction with syntaxin-1 (a component of SNAREs) and enhances the formation of the SNARE complex. Electrophysiological studies using cultured superior cervical ganglion (SCG) neurons revealed that this enhanced formation of the SNARE complex by the PKA-catalyzed phosphorylation of tomosyn increased the fusion-competent readily releasable pool of synaptic vesicles and, thereby, enhanced neurotransmitter release. This mechanism was indeed involved in the facilitation of neurotransmitter release that was induced by a potent biological mediator, the pituitary adenylate cyclase-activating polypeptide, in SCG neurons. We describe the roles and modes of action of PKA and tomosyn in Ca\(^{2+}\)-dependent neurotransmitter release.

Introduction

Synaptic vesicle exocytosis consists of many complicated steps, such as the translocation of vesicles from the reserve pool to the active zone, the docking of vesicles at the active zone, transition from the docking to the priming step (formation of fusion-competent vesicles), and the fusion step (Südhof, 2000). These sequential steps involve a series of protein–protein interactions between the membranes of synaptic vesicles and presynaptic terminals, culminating in the Ca\(^{2+}\)-dependent fusion of the two membranes (Südhof, 2000; Richmond and Broadie, 2002; Rosenmund et al., 2003). Of the many proteins, SNAREs are essential molecules in these processes (Sutton et al., 1998; Weber et al., 1998; Weis and Scheller, 1998; Jahn and Südhof, 1999).

Second messengers regulate protein–protein interactions within the exocytotic machinery and, thereby, modulate neurotransmitter release (Evans and Morgan, 2003). Although the time course between action potential arrival at the nerve terminal and synaptic vesicle fusion is too short for protein phosphorylation/dephosphorylation to exact a direct and acute role in a single round of vesicle exocytosis, protein kinases and phosphatases may have significant effects on subsequent neurotransmitter release events. It is reasonable to speculate that the phosphorylation/dephosphorylation states of synaptic proteins that mediate vesicle exocytosis could regulate the biochemical pathways transiting from the docking to the fusion step. The activation of cAMP-dependent protein kinase (PKA) is known to promote neurotransmitter release from chromaffin cells, hippocampal mossy fiber synapses, and cerebellar parallel fiber synapses by directly acting on the exocytotic machinery (Lonart and Südhof, 1998; Lonart et al., 2003; Nagy et al., 2004). Thus, it is critical to identify the PKA targets that regulate assembly/disassembly of the SNARE complex and the priming of docked vesicles for the elucidation of the molecular mechanisms underlying neurotransmitter release.

Several presynaptic proteins serve as substrates for PKA: α-SNAP (Hirling and Scheller, 1996), Cys string protein (Evans et al., 2001), synapsin I (Jovanovic et al., 2001), snapin (Chheda et al., 2001), syntaphilin (Boczan et al., 2004), RIM1 (Lonart et al., 2003), and SNAP-25 (Risinger and Bennett, 1999; Hepp et al., 2002). In chromaffin cells, two releasable
have shown that Rho-associated coiled-coil–forming kinase (ROCK) that is activated by Rho small G protein phosphorylates syntaxin-1, which increases the affinity of syntaxin-1 for tomosyn and forms a stable tomosyn complex. This results in inhibition of the formation of the SNARE complex during neurotransmission in hippocampal cultured neurons (Sakisaka et al., 2004). The ROCK-catalyzed phosphorylation of syntaxin-1 could act as an “on” switch for tomosyn by enhancing its inhibitory function on the formation of the SNARE complex. Thus, evidence is accumulating that tomosyn acts as a negative regulator for the formation of the SNARE complex, and, thereby, inhibits various vesicle fusion events. However, an “off” switch for tomosyn, which is the molecular regulation that blocks its inhibitory function on the formation of the SNARE complex, remains elusive.

In this study, we show that tomosyn is a target protein for PKA and that the PKA-catalyzed phosphorylation of tomosyn acts as an off switch for tomosyn. It does so by blocking its inhibitory function on the formation of the SNARE complex and, thereby, up-regulating the size of readily releasable synaptic vesicles in neurons.

Results
Phosphorylation of tomosyn by PKA in vitro and in vivo

We first examined the ability of tomosyn to serve as a substrate for PKA in a cell-free assay system using the recombinant proteins. Recombinant maltose-binding protein (MBP)–tagged full-length tomosyn was incubated with the catalytic subunit of PKA in the presence of γ-[32P]ATP and other necessary ingredients. The reaction was terminated by adding an SDS sample buffer and boiling, and each sample was subjected to SDS-PAGE followed by Coomassie brilliant blue (CBB) staining. The gel was then dried and exposed to an X-ray film to detect the incorporation of 32P into tomosyn. 32P was incorporated into tomosyn by PKA, and this incorporation was inhibited by protein kinase peptide inhibitor (PKI), which is a pseudosubstrate PKA inhibitor peptide (Fig. 1 Aa). The incorporation of 32P into tomosyn was time dependent, and 1 mol of phosphate was maximally incorporated into 1 mol of tomosyn (Fig. 1 Ab).

We determined the phosphorylation site of tomosyn by PKA using various tomosyn truncated mutants. When each purified MBP-tagged tomosyn mutant, including aa residues 1–326, 327–600, 601–800, and 801–1116, was incubated with the catalytic subunit of PKA and the exonuclease inhibitor (PKI), the incorporation of 32P into the fragment comprising aa residues 601–800 was efficiently phosphorylated, suggesting that this fragment contains the phosphorylation site by PKA (Fig. 1 Ba). We conducted a sequence search against the Scansite Motif Scanner Program, and several consensus residues for the phosphorylation sites by PKA were predicted in this fragment. We focused on five Ser residues at positions 689, 693, 713, 724, and 745 and generated (by site-directed mutagenesis) tomosyn mutants in which Ser at positions 689, 693, 713, 724, or 745 was replaced by Ala. The incorporation of 32P into the S724A mutant was dramatically decreased, whereas that into other mutants was not markedly reduced (Fig. 1 Bb), indicating that Ser-724 is at least a major phosphorylation site of tomosyn by PKA.
By using Sp-cAMP and forskolin, we examined whether tomosyn is indeed phosphorylated in intact cells in response to cAMP. Sp-cAMP is a nonhydrolyzable and cell-permeable cAMP analogue that induces activation of PKA in intact cells (Scholubbers et al., 1984), whereas forskolin is a potent activator of adenylate cyclase (Seamon et al., 1981). When ³²P-labeled NG108 cells expressing HA-tagged wild-type tomosyn (HA-tomosyn) were stimulated by Sp-cAMP or forskolin, the incorporation of ³²P into tomosyn was significantly enhanced (Fig. 1C). The extent of tomosyn phosphorylation that was stimulated by Sp-cAMP and forskolin was reduced by KT5720 and H-89, which are synthetic chemical PKA inhibitors (Kase et al., 1987; Chijiwa et al., 1990). When ³²P-labeled NG108 cells expressing HA-tomosyn–S724A were stimulated with Sp-cAMP, the incorporation of ³²P into this mutant was not markedly observed. These results indicate that tomosyn is directly phosphorylated by PKA both in vitro and in vivo and that Ser-724 is the major phosphorylation site of tomosyn by PKA.

Reduction by the PKA-catalyzed phosphorylation of tomosyn of its interaction with syntaxin-1 in vitro

We have previously shown that tomosyn interacts with syntaxin-1 in a manner that is competitive with VAMP-2, forms the tomosyn complex, and down-regulates the formation of the SNARE complex (Fujita et al., 1998; Yokoyama et al., 1999; Sakisaka et al., 2004). We examined the effect of the PKA-catalyzed phosphorylation of tomosyn on its interaction with syntaxin-1 in vitro. MBP-tomosyn was either phosphorylated by PKA or left unphosphorylated. We attempted to mimic complete phosphorylation of Ser-724 by mutating it to a negatively charged Asp residue (S724D) or to a noncharged Ala residue (S724A) as a control and analyzed the resulting activity of the mutated proteins interacting with syntaxin-1. MBP-tomosyn, MBP-tomosyn–S724D, or MBP-tomosyn–S724A was incubated with GST–syntaxin-1 beads, and each tomosyn protein that bound to GST–syntaxin-1 beads were separated by centrifugation and subjected to SDS-PAGE followed by CBB staining. The amount of MBP-tomosyn–
S724D that bound to GST-syntaxin-1 was ∼35% in comparison with that of MBP-tomosyn, whereas that of MBP-tomosyn–S724A was almost the same as that of MBP-tomosyn (Fig. 2 C). These results support the aforementioned results that the PKA-catalyzed phosphorylation of tomosyn at Ser-724 decreases its interaction with syntaxin-1 in vitro.

Enhancement of the formation of the SNARE complex by the PKA-catalyzed phosphorylation of tomosyn in vivo

We have previously shown that the expression of tomosyn increases the formation of the tomosyn complex and, thereby, down-regulates the formation of the SNARE complex in NG108 cells (Sakisaka et al., 2004). We examined whether the PKA-catalyzed phosphorylation of tomosyn affects the formation of the SNARE complex. We cultured NG108 cells expressing myc-tomosyn in the presence and absence of Sp-cAMP and immunoprecipitated syntaxin-1 from the lysate of each NG108 cell. Both myc-tomosyn and VAMP-2 were coimmunoprecipitated with syntaxin-1 in both cells, but the amount of coimmunoprecipitated VAMP-2 in the cells that were pretreated with Sp-cAMP was more than that of control cells (Fig. 3). Conversely, the amount of coimmunoprecipitated myc-tomosyn in cells that were pretreated with Sp-cAMP was less than that of control cells. When either myc-tomosyn–S724A or S724D was expressed, the amount of coimmunoprecipitated VAMP-2 in the cells expressing myc-tomosyn–S724D was more than that in the cells expressing myc-tomosyn–S724A. In addition, the amount of coimmunoprecipitated VAMP-2 in the cells expressing myc-tomosyn–S724A or S724D was not increased in response to Sp-cAMP. These results are consistent with those obtained by the in vitro experiments shown in Fig. 2 C. They indicate that the tomosyn complex was more predominantly formed in the cells expressing myc-tomosyn–S724A than in the cells expressing myc-tomosyn–S724D and that, conversely, the SNARE complex was less predominantly formed in the cells expressing myc-tomosyn–S724A than in the cells expressing myc-tomosyn–S724D. These results indicate that the PKA-catalyzed phosphorylation of tomosyn decreases the formation of the tomosyn complex and increases the formation of the SNARE complex in intact cells.
Expression and localization of tomosyn in SCG neurons

Next, we sought to investigate whether the PKA-catalyzed phosphorylation of tomosyn is indeed involved in neurotransmitter release. For this purpose, we used cultured rat superior cervical ganglion (SCG) neurons because a potent biological ligand for activating the endogenous PKA pathway, the pituitary adenylate cyclase-activating polypeptide (PACAP), has been shown to be a potent regulator of neurotransmitter release from sympathetic SCG neurons (May and Braas, 1995). PACAP belongs to the vasoactive intestinal peptide/secretin/glucagon family of peptides, and the 185-amino acid rat precursor molecule is posttranslationally processed to two biologically active α-amidated PACAP27 or 38 peptides (Miyata et al., 1989; Arimura, 1998; Sherwood et al., 2000). PACAPs bind to a PACAP receptor named PAC1 receptor, which is a seven-transmembrane trimeric G protein–coupled receptor that activates adenylate cyclase through the Gs protein subunit, resulting in an elevation of cellular cAMP and the subsequent activation of PKA (May and Braas, 1995). PAC1 receptor has been shown to be highly expressed in sympathetic SCG neurons (May and Braas, 1995). Before undertaking electrophysiological assays, we first examined the expression and localization of tomosyn in SCG neurons. Western blotting showed that tomosyn as well as other SNARE proteins, including syntaxin-1, SNAP-25, and VAMP-2, were expressed in SCG neurons at the same levels as those in the rat cerebrum (Fig. 4 A). Immunofluorescence microscopy showed that the signal for tomosyn partly colocalized with those of syntaxin-1, synaptophysin (synaptic vesicle marker), and Bassoon (active zone marker) at synapses (Fig. 4 B). Tomosyn did not colocalize with the PSD-95 family protein (postsynaptic marker). These results indicate that tomosyn is expressed and localized at the presynaptic terminals of SCG neurons.

Involvement of PKA in neurotransmitter release from SCG neurons

We examined whether PKA modulates neurotransmitter release from SGG neurons. For this purpose, we first examined whether the introduction of cAMP into the presynaptic terminals of SCG neurons enhances the action potential–induced neurotransmitter release, which is measured as the amplitude of excitatory postsynaptic potentials (EPSPs) in synaptically coupled SCG neurons. Injection of cAMP (10 mM in the injection pipette for 5 min) into the presynaptic neurons induced a long-lasting membrane depolarization (2–5 mV for 20–30 min) along with a modest decrease in the amplitudes of the action potential and after hyperpolarization (Fig. 5 Aa). EPSPs that were elicited every 20 s were increased by cAMP (Fig. 5 Ab) with mean amplitudes up to 54 ± 34% during the injection of cAMP and a return to control values by 10–20 min after the injection, which was followed by a further decrease below the baseline (Fig. 5 B). Modest depolarization in the neurons, in which cAMP was injected, was observed, but this did not seem to affect the gating kinetics of N-type Ca^{2+} channels, as described previously (Regan et al., 1991). Consistently, PKA has been shown not to directly modulate N-type Ca^{2+} channels (Bernheim et al., 1991; Zhu and Ikeda, 1993, 1994). Thus, cAMP appears to directly affect the exocytotic machinery. Injection of a higher concentration of cAMP (50 mM in the pipette) induced an increase in EPSP amplitudes, which lasted longer (for 20–30 min) than the injection of 10 mM cAMP, although the maximum increase in EPSP amplitude, 46 ± 24%, was not increased. These results suggest that cAMP produces various changes in presynaptic electrical properties through the activation of PKA and also promotes a persistent increase in acetylcholine release from the presynaptic terminals of SCG neurons. It should be noted that cAMP accelerated the rate of EPSP rise but did not affect the time to reach the peak (Fig. 5 Ac), suggesting that the pool of readily releasable vesicles is increased by the activation of PKA.

It has been proposed that PKA enhances neurotransmitter release by increasing the size of the RRP of synaptic vesicles (Kuromi and Kidokoro, 2000; Lonart et al., 2003; Kaneko and Takahashi, 2004; Nagy et al., 2004) and also by increasing the probability of fusion of synaptic vesicles (Kaneko and Takahashi, 2004). We confirmed, by repetitive local applications of...
Figure 5. Involvement of PKA in neurotransmitter release from SCG neurons. (A) Effects of cAMP on synaptic transmission between SCG neurons. Presynaptic action potentials (Aa) and EPSPs (Ab) from one representative experiment that were recorded in a synapse before and after cAMP injection (10 mM in the pipette for 5 min). (Ac) The first order derivative of EPSPs shown in Ab, representing the rate of rise and fall for EPSPs. (B) 10 (top) or 50 mM CAMP (bottom) in the pipette was injected into the presynaptic neurons at time = 0. Presynaptic neurons were stimulated every 20 s. (C) The RRP size was measured by puff application of 0.5 M sucrose for 2 s every 2–3 s. CAMP (50 mM in the pipette) was introduced into the presynaptic neurons at time = 0. Top three traces are responses to sucrose applications from one representative experiment recorded 2 min before (Cont), 4.5 min after, and 16.5 min after the start of CAMP injection. Bars, 10 mV and 3 s. In the bottom graph, normalized and averaged integral values for sucrose responses are plotted (n = 6; *, P < 0.05 at 1.5 min vs. before injection). (D) Failure of EPSPs after 10 action potentials at 20 Hz recorded in 0.2 mM external Ca2+ solution was examined before white bars and after injection of CAMP (at 50 mM in the pipette; gray bars). (E) Effect of a PKA activator on synaptic transmission between SCG neurons. 500 μM forskolin in the pipette was injected into the presynaptic neurons for 5 min at time = 0. The presynaptic neurons were stimulated every 20 s. (F) Effect of a PKA inhibitor on synaptic transmission between SCG neurons. (Fa) EPSPs from one representative experiment recorded in a synapse before [control] and at 30 min after 10 μM H-89 injection. (Fb) EPSP amplitudes from one representative experiment recorded in a synapse with the presynaptic injections of H-89. No additive reduction was produced by a second injection at a 10-fold higher concentration. (G) Effects of other PKA inhibitors on synaptic transmission between SCG neurons. 10 μM H-89 [Go] or a mixture of 1 μM PKI 6-22 and 1 μM KT5720 (Gb) in the pipette was injected into the presynaptic neurons for 5 min at time = 0. The presynaptic neurons were stimulated every 20 s. (B, E, and G) Normalized EPSP amplitudes were averaged (B and G, n = 4; E, n = 5) and plotted with dots against recording time. The resulting values were smoothed with a moving average algorithm and plotted with a line. (H) Maximum changes in EPSP amplitudes that are shown in E and G were averaged. Error bars represent SEM.

Involvement of the PKA-catalyzed phosphorylation of tomosyn in neurotransmitter release

To investigate the role of the PKA-catalyzed phosphorylation of tomosyn in neurotransmitter release, we analyzed synaptic transmission of SCG neurons by expressing myc-tomosyn, myc-tomosyn–S724A, myc-tomosyn–S724D, or tomosyn small interference RNA (siRNA) in the presynaptic neurons. First, we examined electrophysiologically how many neuron pairs are synaptically coupled. For this purpose, we recorded electrical responses in pairs of neurons by generating action potential trains at 2 Hz in the presynaptic neurons. The incidence of synaptic coupling between control neurons (nontransfected presynaptic neurons) was 64 ± 4.9% (Fig. 6 A). This value was reduced to 40 ± 13 and 44 ± 6.3% by the expression of myc-tomosyn and myc-tomosyn–S724A, respectively, whereas this value was more markedly reduced to 28 ± 11% by the expression of myc-tomosyn–S724A. Similarly, the incidence of synaptic coupling was reduced to 35 ± 10% by expressing tomosyn siRNA, whereas the incidence of synaptic coupling was 61 ± 14% for control neurons expressing control scramble RNA. Then, we measured the size of EPSPs in synaptically coupled neurons. The mean EPSP amplitude for control synapses of nontransfected neuron pairs

a hypertonic solution (Rosenmund and Stevens, 1996), whether PKA is indeed involved in the determination of RRP size. Focal puff application of 0.5 M sucrose to a synaptic pair induced small EPSP events in postsynaptic neurons (Fig. 5 C). The integral value of small EPSPs that were induced by hypertonic challenge reflects the size of the RRP, and, therefore, changes in this integral value after experimental manipulation indicates changes in the RRP size. CAMP increased the integral value by up to 20–25%. In contrast, the release probability of synaptic vesicles was not obviously increased by cAMP in SCG neurons. Failures in postsynaptic responses after 10 presynaptic action potentials at 5, 10, and 20 Hz in the 0.2-mM Ca2+ external solution was not decreased after the injection of CAMP into the presynaptic neurons (Fig. 5 D). These results are consistent with earlier observations in other systems (Kurokami and Kido, 2000; Lontart et al., 2003; Nagy et al., 2004) and suggest that PKA up-regulates neurotransmitter release by increasing the RRP size and does not promote a step at or downstream of the sensing of Ca2+, which triggers synaptic vesicle fusion at the presynaptic terminals of SCG neurons.

To further confirm that PKA is the relevant kinase mediating the effect of cAMP in neurotransmitter release from SCG neurons, we applied various reagents that modulate the PKA signaling pathway, including forskolin, KT5720, and H-89 (Fig. 5, E–H). Forskolin enhanced neurotransmitter release, which is consistent with the effect of cAMP (Fig. 5, E and H). In contrast, H-89 and a combination of PKA inhibitors (KT5720 and the PKA pseudosubstrate PKI 6–22 peptide) inhibited the evoked neurotransmitter release (Fig. 5, F–H). These results indicate that PKA is the relevant kinase for regulating neurotransmitter release from SCG neurons by increasing the RRP size.
was 20 ± 6.8 mV (Fig. 6 B). This value was reduced to 8.2 ± 2.3 and 8.1 ± 2.4 mV by the expression of myc-tomosyn and myc-tomosyn–S724D in the presynaptic neurons, respectively, whereas this value was more markedly reduced to 4.8 ± 0.7 mV by the expression of myc-tomosyn–S724A. Similarly, the mean EPSP value was reduced to 6.7 ± 1.7 mV by the expression of tomosyn siRNA, whereas the mean EPSP value was reduced to 6.7 mV by the expression of myc-tomosyn–S724A. The incidence for each dish is averaged (n = 4–9). The bar graph shows means ± SEM (*, P < 0.05 vs. nontransfected presynaptic neurons). (B) EPSPs from one representative experiment recorded from the synapses between presynaptic neurons that were transfected with the respective tomosyn expression vector and the nontransfected postsynaptic neurons. Means of EPSP amplitudes (n = 4–8; *, P < 0.05 vs. presynaptic neurons that were transfected with myc-tomosyn [WT]). (C) Expression levels of various tomosyn mutants and the effect of tomosyn knockdown by the RNA interference method in SCG neurons. SCG neurons were comicroinjected with fluorescein dextran as a morphological marker along with the same various tomosyn mutant (left) or siRNA (right) expression vectors that were used in A and were cultured for 40 h. The transfected cells were identified by microinjected fluorescein dextran (green), and the extent of expression of various tomosyn mutants was examined by immunostaining of tomosyn (red). The cell nuclei and plasma membrane borders of the neighboring nontransfected postsynaptic neurons are marked with dashed lines. N, nucleus. Arrows indicate the presynaptic terminals. Bars, 10 μm. (bottom) Quantification of relative fluorescence intensities of tomosyn at the presynaptic terminal. The results shown are representative of three independent experiments. Error bars represent SEM.

Figure 6. Involvement of the PKA-catalyzed phosphorylation of tomosyn in neurotransmitter release from SCG neurons. (A) Incidence of synaptic coupling between the nontransfected pre- and postsynaptic neurons (control) and between the presynaptic neurons that were transfected with myc-tomosyn (WT), myc-tomosyn–S724A (S724A), myc-tomosyn–S724D (S724D), tomosyn siRNA, or scramble RNA and the nontransfected postsynaptic neurons. The incidence for each dish is averaged (n = 4–9). The bar graph shows means ± SEM (*, P < 0.05 vs. nontransfected presynaptic neurons). (B) EPSPs from one representative experiment recorded from the synapses between presynaptic neurons that were transfected with the respective tomosyn expression vector and the nontransfected postsynaptic neurons. Means of EPSP amplitudes (n = 4–8; *, P < 0.05 vs. presynaptic neurons that were transfected with myc-tomosyn [WT]). (C) Expression levels of various tomosyn mutants and the effect of tomosyn knockdown by the RNA interference method in SCG neurons. SCG neurons were comicroinjected with fluorescein dextran as a morphological marker along with the same various tomosyn mutant (left) or siRNA (right) expression vectors that were used in A and were cultured for 40 h. The transfected cells were identified by microinjected fluorescein dextran (green), and the extent of expression of various tomosyn mutants was examined by immunostaining of tomosyn (red). The cell nuclei and plasma membrane borders of the neighboring nontransfected postsynaptic neurons are marked with dashed lines. N, nucleus. Arrows indicate the presynaptic terminals. Bars, 10 μm. (bottom) Quantification of relative fluorescence intensities of tomosyn at the presynaptic terminal. The results shown are representative of three independent experiments. Error bars represent SEM.

Involvement of the PKA-catalyzed phosphorylation of tomosyn in maintaining a pool of readily releasable synaptic vesicles during repetitive stimulation

We examined whether the reduction of neurotransmitter release by the expression of myc-tomosyn, myc-tomosyn–S724A, or myc-tomosyn–S724D is caused by a depletion of synaptic vesicles that are primed for action potential–triggered fusion. For this purpose, 10 action potentials were generated in presynaptic neurons expressing myc-tomosyn, myc-tomosyn–S724A, or myc-tomosyn–S724D, and the evoked neurotransmitter release after each train of action potentials at varying stimulation frequencies was measured (Fig. 7 A). Experiments were performed in a low external Ca²⁺ solution to avoid spike generation in the postsynaptic neurons. Control synapses produced EPSPs after each of 10 action potentials that were generated at 5, 10, and 20 Hz in 1.0 and 0.5 mM external Ca²⁺ solutions but not in 0.2 mM external Ca²⁺ solution (Fig. 7, A–Bb). In contrast, EPSP failures were recorded in synapses between the presynaptic neurons expressing myc-tomosyn and nontransfected postsynaptic neurons even in 1.0 and 0.5 mM external Ca²⁺ solutions (Fig. 7 Bc). EPSP failure after a 20-Hz train in 0.5 mM Ca²⁺ solutions (Fig. 7 Bc). EPSP failure after a 20-Hz train in 0.5 mM Ca²⁺ solutions (Fig. 7 Bc). EPSP failure after a 20-Hz train in 0.5 mM Ca²⁺ solutions (Fig. 7 Bc). EPSP failure after a 20-Hz train in 0.5 mM Ca²⁺ solutions (Fig. 7 Bc). EPSP failure after a 20-Hz train in 0.5 mM Ca²⁺ solutions (Fig. 7 Bc). EPSP failure after a 20-Hz train in 0.5 mM Ca²⁺ solutions (Fig. 7 Bc). EPSP failure after a 20-Hz train in 0.5 mM Ca²⁺ solutions (Fig. 7 Bc). EPSP failure after a 20-Hz train in 0.5 mM Ca²⁺ solutions (Fig. 7 Bc).
Figure 7. Involvement of the PKA-catalyzed phosphorylation of tomosyn in maintaining a pool of readily releasable synaptic vesicles during repetitive stimulation. EPSPs after 10 action potentials elicited in the nontransfected presynaptic neurons (control) and the presynaptic neurons that were transfected with myc control vector (vector), myc-tomosyn (WT), myc-tomosyn–S724A (S724A), or myc-tomosyn–S724D (S724D). [A] Top trace shows presynaptic action potentials elicited at 20 Hz. Other traces show postsynaptic potentials after repetitive action potentials from one representative experiment that was recorded in synapses between the presynaptic neurons that were transfected with each tomosyn expression vector and the nontransfected postsynaptic neurons. Electrical responses are recorded in 0.5 mM CaCl$_2$-containing modified Krebs’ solution. *, Action potentials are generated in the nontransfected neurons and followed by three EPSPs overlapping with hyperpolarizations. (B) Failures of EPSPs after 10 action potentials at 5, 10, and 20 Hz generated in the presynaptic neurons that were transfected with the respective tomosyn expression vectors were recorded in 1, 0.5, and 0.2 mM external Ca$^{2+}$ solutions ($n = 5–6$; *, $P < 0.05$ vs. the presynaptic neurons that were transfected with myc control vector). Error bars represent SEM.

was also observed in synapses between the presynaptic neurons expressing myc-tomosyn–S724A or –S724D and the nontransfected postsynaptic neurons (Fig. 7, Bd and Be). The EPSP waveform became entangled by asynchronous responses during trains of action potentials at 20 Hz and occasionally at 10 Hz in synapses between the presynaptic neurons expressing myc-tomosyn–S724A or –S724D and the nontransfected postsynaptic neurons (Fig. 7 A). These asynchronous response profiles were not prominent in synapses between the presynaptic neurons expressing myc-tomosyn and the nontransfected postsynaptic neurons. Thus, neurotransmitter release from the presynaptic neurons, in which the phosphorylation of tomosyn was prevented, was not synchronized to each action potential during high, frequent presynaptic activity. Instead, an asynchronous mode of neurotransmitter release was induced. These results suggest that the PKA-catalyzed phosphorylation of tomosyn is likely to play an important role in maintaining a pool of readily releasable synaptic vesicles that are synchronized to each action potential during repetitive presynaptic activity.

Involvement of the PKA-catalyzed phosphorylation of tomosyn in determination of the RRP size

Next, we determined whether the PKA-catalyzed phosphorylation of tomosyn is involved in the determination of RRP size. cAMP was injected into the presynaptic neurons expressing myc-tomosyn, myc-tomosyn–S724A, myc-tomosyn–S724D, or tomosyn siRNA. EPSP amplitudes were increased up to 45 ± 18% during the injection of cAMP and persisted for >20 min in synapses between the presynaptic neurons expressing myc-tomosyn and the nontransfected postsynaptic neurons (Fig. 8, Aa and B). In contrast, the increase in EPSP amplitude was significantly less in synapses between the presynaptic neurons expressing myc-tomosyn–S724A and the nontransfected postsynaptic neurons (Fig. 8, Ab and B). In synapses between presynaptic neurons expressing myc-tomosyn–S724D and nontransfected postsynaptic neurons, EPSP amplitudes slowly increased after the injection of cAMP (Fig. 8, Ac and B). Apparently, the time course of enhancement of neurotransmitter release by cAMP in the presynaptic neurons expressing myc-tomosyn–S724D was different from that of the presynaptic neurons expressing myc-tomosyn (Fig. 8, Aa and Ac). The increase in EPSP amplitude that was caused by the injection of cAMP was significantly blocked by the expression of tomosyn siRNA in the presynaptic neurons (Fig. 8, Ad and B), whereas the increase was obviously observed in synapses between the presynaptic neurons expressing control scramble RNA and the nontransfected postsynaptic neurons (Fig. 8 Ae). These results suggest that PKA up-regulates neurotransmitter release via the phosphorylation of tomosyn at S724, resulting in an increase in the fusion-competent RRP of vesicles that are synchronized to the arrival of action potentials.

Involvement of the PKA-catalyzed phosphorylation of tomosyn in the PACAP-induced facilitation of neurotransmitter release

In the last set of experiments, we investigated the physiological significance of the PKA-catalyzed phosphorylation of tomosyn in neurotransmitter release from SCG neurons by using PACAP. Before undertaking electrophysiological assays, we first examined the localization of PAC1 receptor in SCG neurons. Immunofluorescence microscopy showed that the signal for PAC1 receptor partly colocalized with those of tomosyn, syntaxin-1, synaptophysin, and Bassoon at synapses (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200504055/DC1). PAC1 receptor did not colocalize with the PSD-95 family protein. These results indicate that PAC1 receptor is expressed and localized at the presynaptic terminals of SCG neurons.

We then examined whether the PKA-catalyzed phosphorylation of tomosyn is involved in the PACAP-induced facilitation of neurotransmitter release. To investigate the role of PACAP at presynaptic terminals in SCG neurons, we focally applied PACAPs to presynaptic SCG neurons. Focal puff application of PACAP27 or 38 (1 nM in the pipette for 0.5 s) markedly increased EPSP amplitudes and prolonged the time course of EPSP waveforms (Fig. 9 Aa). EPSPs that
PKA-K72H (Iyer et al., 2005), or a mixture of PKA catalytic subunits with myc-tomosyn–S724A (S724A), myc-tomosyn–S724D (S724D), or scramble RNA and nontransfected postsynaptic neurons. In the presence of cAMP, PACAP was injected into the presynaptic neurons at time 0 (50 mM in the pipette). Presynaptic neurons were stimulated every 20 s. EPSP amplitudes were averaged (n = 4–6) and plotted with dots. The smoothed value with a moving average algorithm was plotted with a line. (B) Bar graphs show increases in the EPSP amplitude by cAMP, which is shown in A. Each bar shows the smoothed values of EPSP amplitudes at 10, 15, and 20 min after injection. *, P < 0.05 versus presynaptic neurons that were transfected with myc-tomosyn (WT). Error bars represent SEM.

Figure 9. Involvement of the PKA-catalyzed phosphorylation of tomosyn in the PACAP-induced facilitation of neurotransmitter release. (A) Effects of PACAP peptides on synaptic transmission between SCG neurons. (Aa) EPSPs from one representative experiment recorded in a synapse before (control) and after PACAP27 puff application (1 nM in the pipette for 0.5 s). (Ab) 1 nM PACAP27 in the pipette was puff applied onto the presynaptic neurons at time 0 for 0.5 s. (Ac) 1 nM PACAP38 was applied as described in Ab. (B) Maximum increases in EPSP amplitudes were averaged (n = 4). (C) Mean amplitudes of EPSPs that were evoked by stimuli of the presynaptic neurons transfected with PKA-K72H or PKA siRNA (n = 4–5; *, P < 0.05 versus nontransfected synapses). (D) Effects of PACAP27 on EPSP amplitudes that were evoked by stimuli of the presynaptic neurons transfected with PKA-K72H, PKA siRNA, or myc-tomosyn–S724A. PACAP27 was applied as described in Ab. (A and D) Presynaptic neurons were stimulated every 20 s. EPSP amplitudes were averaged (n = 4) and plotted with dots. The resulting values were smoothed with a moving average algorithm and plotted with a line. (E) Maximum increases in averaged EPSP amplitudes after PACAP27 application at 35 min after the application for nontransfected cells, 3 min for PKA-K72H, 30 min for PKA siRNA, and 19 min for myc-tomosyn–S724A expressing synapses (n = 4; *, P < 0.05 versus nonexpressing synapses). Error bars represent SEM. (F) A model of a role for tomosyn in the long-lasting PACAP-induced facilitation of neurotransmitter release. PACAP binds to PAC1 receptor, which is a seven-transmembrane trimeric G protein-coupled receptor that activates adenylate cyclase through the Gs protein subunit, resulting in an elevation of cellular cAMP and subsequent activation of PKA. The PKA-catalyzed phosphorylation of tomosyn, which is the off switch for tomosyn, reduces its interaction with syntaxin-1 and enhances formation of the SNARE complex, resulting in an increase in the RRP size for the action potential-evoked neurotransmitter release. Thus, the PKA-catalyzed phosphorylation of tomosyn is involved in the physiological trimeric G protein-coupled receptor’s signaling pathway and may play a role in long-lasting synaptic modulation or plasticity. AC, adenylate cyclase.

were elicited every 20 s were increased by PACAP27 and 38 (Fig. 9, Ab and Ac), with the mean of peak amplitudes increased to 75 ± 27 and 66 ± 16% of baseline control responses, respectively (Fig. 9 B). These results indicate that low concentrations of PACAP peptides facilitate neurotransmitter release, which is consistent with a previous study for neuropeptide Y release (May and Braas, 1995). In concordance with cAMP injections into the presynaptic neurons, peptides slightly depolarized the presynaptic membrane (0–3 mV for 30–40 min; not depicted); however, this modest depolarization does not affect the gating kinetics of N-type Ca2+ channels (Regan et al., 1991). Then, we examined whether PKA is involved in the PACAP-induced facilitation of neurotransmitter release by expressing a kinase-dead mutant, PKA-K72H (Iyer et al., 2005), or a mixture of PKA catalytic subunits (PKA Cα and Cβ) siRNAs (Dumaz and Marais, 2003) in the presynaptic neurons. These inhibitory reagents reduced the EPSP amplitude to 8.9 ± 2.1 or 13 ± 0.6 mV in comparison with the value of 20 ± 1.1 mV in nontransfected neuron pairs (Fig. 9 C) and blocked the PACAP27-induced facilitation of neurotransmitter release (Fig. 9, Da and Db). Myc-tomosyn–S724A also reduced the PACAP27-induced facilitation of neurotransmitter release, confirming the involvement of the PKA-catalyzed phosphorylation of tomosyn in the PACAP-induced facilitation of neurotransmitter release (Fig. 9 Dc). The maximum increase in the mean EPSP amplitude that was caused by PACAP27 (57 ± 30% at 35 min after the application) was significantly reduced by 12 ± 18 (at 31 min; P < 0.05), 30 ± 20 (at 30 min), and 25 ± 8% (at 19 min) with...
the expression of PKA-K72H, PKA siRNA, and myc-tomosyn-S724A, respectively (Fig. 9 E). These results suggest that PACAP induces the facilitation of neurotransmitter release through the PKA-catalyzed phosphorylation of tomosyn in SCG neurons and regulates synaptic transmission efficiency in the autonomic nervous system.

Discussion

First, we have shown in this study that tomosyn is phosphorylated by PKA in vitro and in vivo. The major phosphorylation site is Ser-724, which locates in the linker region between the NH₂-terminal half WD40 repeat domain and the COOH-terminal coiled-coil VAMP homology domain. We have then shown that the PKA-catalyzed phosphorylation of tomosyn inhibits its interaction with syntaxin-1, resulting in the enhanced formation of the SNARE complex in vivo. The COOH-terminal coiled coil VAMP homology domain is responsible for the interaction with syntaxin-1 (Yokoyama et al., 1999; Hatsuzawa et al., 2003). Through this VAMP homology domain, tomosyn competes with VAMP-2 for interaction with syntaxin-1 and, thereby, prevents formation of the SNARE complex (Yokoyama et al., 1999; Hatsuzawa et al., 2003). However, our previous studies have shown that this VAMP homology domain is necessary, but not sufficient for, the high affinity interaction of tomosyn with syntaxin-1 (Masuda et al., 1998; Yokoyama et al., 1999). Therefore, the linker region may make the ternary structure of the VAMP homology domain more sensitive to syntaxin-1. The phosphorylation of this linker region may reduce its positive effect on the VAMP homology domain and decrease the affinity of the interaction of tomosyn with syntaxin-1. Although further studies are necessary to elucidate the molecular mechanism of the effect of the linker region, the PKA-catalyzed phosphorylation of the linker region may act as an intramolecular off switch for tomosyn by blocking its inhibitory effect on the formation of the SNARE complex.

Second, we have shown that the PKA-catalyzed phosphorylation of tomosyn is involved in neurotransmitter release from SCG neurons. In adrenal chromaffin cells, the PKA-catalyzed phosphorylation of SNAP-25 has been proposed to regulate the priming/depriming of secretory vesicles at a slow rate-limiting step before the final fast priming step and Ca²⁺-dependent fusion (Nagy et al., 2004). Therefore, another unidentified PKA target has been postulated to be involved in regulation of the priming step that is associated with the quickly releasable pool, which may control the availability of readily releasable vesicles in nerve terminals (Heidelberger and Matthews, 2004). Our results suggest that tomosyn is a candidate PKA target for this penultimate priming step. Upon high, frequent stimulation, the expression of tomosyn S724A (a mutant that is resistant to the phosphorylation of PKA) or S724D (a mutant mimicking the phosphorylated form of tomosyn) increases the EPSP failure rate and causes the appearance of asynchronous EPSP waveforms, suggesting that the mobilization of readily releasable vesicles is strongly reduced when the PKA-catalyzed phosphorylation of tomosyn Ser-724 is impaired. An increase in asynchronous release upon repetitive stimulation has also been observed when the interaction of the synprint site in N-type Ca²⁺ channels with the SNARE complex is disrupted by synprint peptides (Mochida et al., 1996). It is likely that preventing the formation of the SNARE complex reduces its interaction with N-type Ca²⁺ channels that are required for synchronous neurotransmitter release. Although further studies are required for our understanding of the molecular mechanism of vesicle priming, we propose that the PKA-catalyzed phosphorylation of tomosyn plays an important role in supplying readily releasable synaptic vesicles to a population of synchronized vesicles, which are able to respond to each action potential during repetitive presynaptic activity. Altogether, our results indicate that tomosyn is a physiologically significant PKA target that controls neurotransmitter release through regulation of the formation of the SNARE complex and vesicle priming.

Third, we have shown that the knockdown of tomosyn decreases neurotransmitter release from SCG neurons, indicating that tomosyn plays both positive and negative roles in neurotransmitter release. The exact reason for these apparently inconsistent data is not known, but one explanation is that the formation of the tomosyn complex may be a prerequisite for the formation of the SNARE complex by analogy with the role of Munc-18 in the formation of the SNARE complex (Gerst, 2003). Because the amount of tomosyn is less than that of syntaxin-1, tomosyn may catalytically facilitate the formation of the SNARE complex. This idea requires the process in which tomosyn is replaced by VAMP-2 to form the SNARE complex. The PKA-catalyzed phosphorylation of tomosyn may be involved in this process for releasing tomosyn from syntaxin-1 and SNAP-25. Another possible explanation is that tomosyn affects another regulatory component besides SNAREs, such as a cytoskeletal network, in the exocytosis of neurotransmitters. Tomosyn is a member of the Lgl family (Katoh and Katoh, 2004). Lgl is part of the cytoskeletal network and is associated with nonmuscle myosin II heavy chain (Strand et al., 1994), which localizes at the presynaptic side of synaptic junctions (Beach et al., 1981) and is involved in neurotransmitter release (Mochida et al., 1994a). Recently, it was found that the aPKC-catalyzed phosphorylation of Lgl inhibits the interaction with myosin II and allows an autoinhibitory intramolecular interaction of the NH₂-terminal portion of Lgl with the COOH-terminal portion (Betschinger et al., 2005). Similar to Lgl, tomosyn may interact with myosin II under the control of the PKA-catalyzed phosphorylation of tomosyn, and this interaction may be involved in the exocytosis of neurotransmitters.

Finally, we have shown the physiological significance of the PKA-catalyzed phosphorylation of tomosyn in neurotransmitter release from SCG neurons by using a potent biological ligand to activate the endogenous PKA pathway (PACAP). Consistent with previous studies, locally applied PACAP increases EPSP amplitude along with a slight membrane depolarization that is similar to the introduction of cAMP or forskolin into the presynaptic nerve terminals of SCG neurons (May et al., 1998; Beaudet et al., 2000). However, the effect of PACAP lasts longer than that of cAMP or forskolin. Inhibition of the PKA signaling pathway by the expression of kinase-dead
PKA-K72H or a mixture of PKA catalytic subunit (PKA Ca and Cβ) siRNAs significantly reduces the long-lasting PACAP-induced facilitation of neurotransmitter release. The expression of tomosyn-S724A (a mutant that is resistant to the phosphorylation of PKA) also reduces the long-lasting PACAP-induced facilitation of neurotransmitter release. The activity of tomosyn may depend on the level of PKA activation in the presynaptic SCG neurons. In fact, the basal level of PKA activity varies at different synapses (Chavis et al., 1998). In the squid giant synapse, a high resting level of PKA activity tonically enhances neurotransmitter release that is elicited by single action potentials, suggesting that the function of PKA at this synapse is to set the initial efficacy of synaptic transmission (Hilfiker et al., 2001). In addition, PKA has been shown to be involved in the exocytosis of various secretory systems (Evans and Morgan, 2003). Thus, the extent of PKA-catalyzed phosphorylation of tomosyn may modulate exocytosis not only in SCG neurons but also in various secretory systems that under the control of the physiological signaling pathway, such as a trimeric G protein–coupled receptor’s signaling pathway (Fig. 9F).

We have previously shown that ROCK activated by Rho small G protein phospholyses syntaxin-1, which increases the affinity of syntaxin-1 for tomosyn and forms a stable complex with tomosyn, resulting in the inhibition of SNARE complex formation during neurite extension (Sakisaka et al., 2004). The ROCK-catalyzed phosphorylation of syntaxin-1 could act as an on switch for tomosyn by enhancing its inhibitory function on the formation of the SNARE complex. Conversely, the PKA-catalyzed phosphorylation of tomosyn could act as an off switch for tomosyn by blocking its inhibitory function on formation of the SNARE complex. Although the involvement of the ROCK-catalyzed phosphorylation of syntaxin-1 in neurotransmitter release needs to be addressed, tomosyn is an important SNARE regulatory protein in neurotransmitter release, whose activity is regulated via well-known signal transduction pathways.

Materials and methods

Construction of expression vectors
cDNAs encoding various fragments of rat medium-sized tomosyn (aa 1–326, 327–600, 601–800, and 801–1,116) were subcloned into pMAL-C2 (New England Biolabs, Inc.). Tomosyn fragment (aa 601–800) mutants, in which Ser at aa residues 689, 693, 713, 724, or 745 was replaced with Ala, were generated using a Site-Directed Mutagenesis Kit (Stratagene). Expression vectors for full-length tomosyn were constructed in pcMV-HA, pEF-BOS-myc, and pFastBac1-MBP by using standard molecular biology methods. pFastBac1-MBP was constructed with a baculovirus transfer vector, pFastBac1 (Invitrogen), to express a fusion protein with MBP-tomosyn in SCG neurons. The lysate of NG108 cells was prepared by using a lysis buffer (20 mM Heps-NaOH, pH 7.4, 90 mM KOAc, 2 mM Mg(OAc)2, 0.5 mM EGTA, 50 mM NaF, 2 mM Na3VO4, and 1% CHAPS; Fujita et al., 1998). The lysates were incubated with an antisyntaxin-1 mAb at 4°C for 2 h. The immune complexes were precipitated with protein G-Sepharose beads (GE Healthcare) at 4°C for 1 h, washed three times with phosphate-free PBS, and incubated for 2 h. The immunoprecipitates were subjected to SDS-PAGE followed by primary Abs, anti-β-actin, and anti-tomosyn Abs (Invitrogen) to detect primary Abs in immunofluorescence microscopy of SCG neurons.

Phosphorylation of tomosyn by PKA in a cell-free system
Phosphorylation of tomosyn was performed in 50 μl of a reaction mixture containing 50 mM Tris-HCl, pH 7.5, 7 mM MgCl2, 1 mM DTT, 0.06% CHAPS, 1 mM EDTA, 50 μM γ[32P]ATP (0.5–1.2 × 104 cpm/μmol; GE Healthcare), and 25 units of the catalytic subunit of PKA (Promega) at 30°C in the presence or absence of 2 mM pseudosubstrate PKI (Promega). The reaction was stopped by the addition of SDS sample buffer and was boiled for 5 min. Each sample was subjected to SDS-PAGE followed by protein staining with CBB and autoradiography. Relevant gel slices were excised, and scintillation was counted to determine the radioactive incorporation into tomosyn.

Phosphorylation of tomosyn in intact cells
Recombinant tomosyn-1-HA fusion protein was produced in HEK293T cells using baculovirus-infecting bacmid DNA. The antitomosyn pAb was prepared as described previously (Fujita et al., 1998). AlexaFluor-conjugated secondary Abs (Invitrogen) were used to detect primary Abs in immunofluorescence microscopy of SCG neurons.

Assay for the interaction of tomosyn with syntaxin-1
40 pmol MBP-tomosyn was fully phosphorylated by 25 units of the catalytic subunit of PKA. Phosphorylated tomosyn was incubated with 30 pmol GST–syntaxin-1 that was bound to 10 μl glutathione beads in the presence of 1% CHAPS at 4°C for 30 min followed by centrifugation and extensive washing. As a control, 40 pmol MBP-tomosyn was incubated under the same conditions as described above except that γ[32P]ATP was excluded. The intensity of the bands in SDS-PAGE was determined by densitometry.

Assay for the formation of tomosyn and SNARE complexes
The lystate of NG108 cells was prepared by using a lysis buffer (20 mM Heps-NaOH, pH 7.4, 90 mM KOAc, 2 mM Mg(OAc)2, 0.5 mM EGTA, 50 mM NaF, 2 mM Na3VO4, and 1% CHAPS; Fujita et al., 1998). The lysates were incubated with an antisyntaxin-1 mAb at 4°C for 2 h. The immune complexes were precipitated with protein G-Sepharose beads (GE Healthcare) at 4°C for 1 h, washed with the lysis buffer, resolved in 5–20% gradient SDS-PAGE, and analyzed by immunoblotting with anti-tomosyn, anti–VAMP-2, anti–SNAP-25, and anti–syntaxin-1 pAbs.

Culture of SCG neurons
Postnatal day 7 Wistar ST rats were decapitated under diethyl ether anesthesia according to Physiological Society of Japan guidelines. Isolated SCG neurons were maintained in culture for 5–6 wk as described previously (Mochida et al., 1994b; Mochida, 1995). In brief, SCGs were dissected, desheathed, and incubated with 0.5 mg/ml collagenase (Worthington Biochemical) or 2 mg/ml trypsin (Promega) for 40 min at 37°C, washed with medium (L-15; Invitrogen) containing 0.10 mM HEPES, 0.33 mM glucose, and 25 units of the catalytic subunit of PKA (Promega) at 37°C for 20 min followed by centrifugation and extensive washing. As a control, 40 pmol MBP-tomosyn was incubated under the same conditions as described above except that γ[32P]ATP was excluded. The intensity of the bands in SDS-PAGE was determined by densitometry.

Immunocytochemistry
SCG neurons were fixed with 4% PFA for 30 min or acetone for 5 min. After washing with PBS, the cells were permeabilized with 0.25% Triton X-100 in PBS for 5 min. Nonspecific binding was blocked with 2% BSA in PBS. After washing, the cells were incubated with primary Abs that were diluted in Block Ace (for 2 h. The cells were incubated with primary Abs that were diluted in Block Ace for 2 h, followed by secondary Abs. The stained cells were observed with a confocal laser microscope.
Expression and knockdown of PKA or tomosyn in SCG neurons

The mammalian expression vector pBS-H1 (Yamada et al., 2005) was used for the expression of siRNA. The following inserts were used: the PKA Cα gene–specific insert was a 21-nucleotide sequence corresponding to nt 885–905 (5'-AAGTGTTTGGCACAACGTAC-3') of PKA Cα cDNA, and the PKA Cβ gene–specific insert was nt 25–45 (5'-AAGAAGTTCCTAGCAAAAGC-3') of PKA Cβ cDNA, which was separated by a 10-nucleotide noncomplementary spacer (5'-TGATACCCG-3') from the reverse complement of the same 21-nucleotide sequence (1-21 of the reverse complement) (Bernheim et al., 2003). An expression vector for tomosyn siRNA was constructed (Sakisaka et al., 2004). Various tomosyn or PKA expression vectors and the tomosyn or PKA siRNA expression vector were microinjected into the nuclei of SCG neurons through a microglass pipette along with 10 kD fluorescein dextran (Invitrogen) as described previously (Mochida et al., 2003). Entry of the constructs into the cell was monitored by fluorescence intensity in the nucleus or that of the dye in the cell body. The cells were maintained at 37°C in a 95% air/5% CO2 humified incubator for 40–70 h, and the injected neurons were identified with an inverted microscope (Diaphot 300; Nikon) equipped with an epifluorescence unit and a fluorescence microscope system (Arugas;Hi/SCA; Hamamatsu Photonics). The injected neurons were examined as the presynaptic neurons in paired neuronal recordings.

Synaptic transmission between SCG neurons

EPSPs were recorded as described previously (Mochida et al., 1994a). Conventional intracellular recordings were made from two neighboring neurons by using microelectrodes filled with 1 M KCl (70–90 MΩ). EPSPs were recorded from a nontransfected neuron, whereas action potentials were generated in the transfected neuron with various tomosyn expression vectors or between neurons that were transfected with various tomosyn expression vectors or between neurons that were transfected with scramble RNA. The injected neurons were examined as the presynaptic neurons in paired neuronal recordings.

Online supplemental material

Fig. S1 shows the localization of PACAP receptor in SCG neurons. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200504055/DC1.

References


