Synapsin I-associated Phosphatidylinositol 3-Kinase Mediates Synaptic Vesicle Delivery to the Readily Releasable Pool*

Received for publication, March 7, 2003, and in revised form, May 7, 2003
Published, JBC Papers in Press, May 16, 2003, DOI 10.1074/jbc.M302386200

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Maintaining synaptic transmission requires replenishment of docked synaptic vesicles within the readily releasable pool (RRP) from synaptic vesicle clusters in the synapsin-bound reserve pool. We show that synapsin forms a complex with phosphatidylinositol 3-kinase (PI 3-kinase) in intact nerve terminals and that synapsin-associated kinase activity increases on depolarization. Disruption of either PI 3-kinase activity or its interaction with synapsin inhibited replenishment of the RRP, but did not affect exocytosis from the RRP. Thus we conclude that a synapsin-associated PI 3-kinase activity plays a role in synaptic vesicle delivery to the RRP. This also suggests that PI 3-kinase contributes to the maintenance of synaptic transmission during periods of high activity, indicating a possible role in synaptic plasticity.

A typical nerve terminal in the central nervous system contains about 200–250 synaptic vesicles (SVs). These can be functionally divided into a small readily releasable pool (RRP) and a large reserve pool. The RRP contains less than 5% of total nerve terminal SVs and is defined morphologically as pre-docked SVs at the active zone and functionally as SVs that are primed and immediately available for the initial rapid phase of neurotransmitter release (1). The remainder constitutes the reserve pool, which ensures that a continual supply of SVs is available for delivery to the RRP and is drawn upon to maintain neurotransmission during periods of intense or prolonged stimulation (2–4). The reserve pool SVs surround the active zone and are clustered together in an actin-based cytoskeletal matrix via an interaction with synapsins (3, 5).

There are five synapsin proteins (Ia, Ib, Ia, Ib, and III) from three genes (I, II, and III) (6), which maintain the reserve pool SVs as a cluster and prevent their free dispersal within the terminal. Synapsins I and II control SV replenishment of the RRP, because conditions that disrupt their function result in dispersal or depletion of SV clusters near the active zone and enhance synaptic depression (3, 5, 7). In contrast, disruption of the much less abundant synapsin III does not affect SV clusters and reduces synaptic depression (8). SV liberation from clusters within the active cytomatrix is mediated by the multi-site phosphorylation of synapsin I and II, which decreases their affinity for SVs or actin (6). Disruption of synapsin I phosphorylation sites thus reduces the number of SVs undergoing exocytosis (9). It is unknown how liberated SVs translocate to the RRP, and the mechanisms governing entry into the RRP (docking/priming) are incompletely understood (10).

Synapsin I has a C-terminal proline-rich domain that interacts with src homology 3 (SH3) domains present in a large number of proteins, including the p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase) (6, 11). A precise role for PI 3-kinase in nerve terminal function has not been established (12–14), despite that its lipid products play numerous roles the actin cytoskeleton and in vesicle trafficking (15, 16). Synapsin I also connects vesicle traffic with the actin cytoskeleton and the synaprasaphin-in vitro interaction suggests that PI 3-kinase activity might play a role in SV recycling if it occurred in nerve terminals. We examined glutamate release from isolated nerve terminals (synaptosomes) and hippocampal CA1 neurons, and our results implicate synapsin-associated PI 3-kinase activity in the delivery of SVs from the reserve pool to the RRP during periods of high activity or prolonged stimulation.

EXPERIMENTAL PROCEDURES

Materials—Wortmannin, LY294002 and protein G were from Sigma, Glutathione-Sepharose from Amersham Biosciences, wConotoxin-GVIA from Bachem (Saffron-Walden, UK), wAgatoxin-IVA from the Peptide Institute (Osaka, Japan), p85 antibody from Santa Cruz Biotechnology (Santa Cruz, CA), synapsin I antibody from Upstate Biotechnology (Lake Placid, NY), and the polyclonal synapsin antibody for immunoprecipitations from Mike Browning (Denver, CO). Synthetic peptides Syn I585–600, GAPPARPPASPSQ, Syn I566–577, SISGAPPKVS, and Syn I566–580, RGQFPQKPPGAPG were synthesized by Auspep (Melbourne, Australia). Penetratin heptapeptide versions (17) of the Syn I566–580, (RRMKWKQ-RGQFPKQFPAGIP or β-adap- tim AP-2 β2 δ4 (RRMKWKQ-QGDLGLDLSNLGPGPVYPQ), were synthesized by Genemed Synthesis (San Francisco, CA). Syn I566–580 showed no homology to synapsin II or III. HF-2035 was a gift from H. Hidaka (Nagoya, Japan). The SH3 domain-containing constructs were: Grb2 p(GEx2-224) (GRMKWKQ-QGDLGLDLSNLGPGPVYPQ), Grb2 (pGEx2-224) from Roger Daly (Sydney, Australia); p85 p(GEx2-2T) plasmid encoding the SH3 domain amino acids 2–83 of bovine p85-PI 3-kinase from Tony Dawson (Toronto, Ontario, Canada); or amphiphysin I (pGEx2-2T-Amp 1 SH3 domain, amino acids 588–695) and amphiphysin II (pGEx2-2T-Amp

*This work was supported by grants from The Wellcome Trust (Ref. 062841), the Australian National Health and Medical Research Council, a University of Sydney Postgraduate Research Scholarship, and a University of Edinburgh Medical Faculty Scholarship. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ These abbreviations used are: SV, synaptic vesicle; RRP, readily releasable pool; PI 3-kinase, phosphatidylinositol 3-kinase; SH3, src homology 3; A1, amphiphysin I; A2, amphiphysin II; GST, glutathione S-transferase; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; AP-2, adaptor protein-2; SH3, glutathione; EPSC, excitatory postsynaptic current; Pk, 3,4,5-P5, phosphatidylinositol 3,4,5-trisphosphate; IP, immunoprecipitation.

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Amph 2 SH3 domain, amino acids 516–612) from Pietro De Camilli (Yale, CT).

Synaptosome 32P Labeling and Pull-downs—Synaptosomes from rat brain were labeled with 32P (18, 19), preincubated for 15 min at 37 °C in the absence or presence of drugs and depolarized with 30 mM KCl (S1). Synaptosomes were resolved in the presence or absence of drug for 7 min before a second KCl stimulus (S2). Synaptosomes (control, S1, depolarization, and S2) were lysed in 25 mM Tris, pH 7.4, containing 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 2 mM EGTA, 50 mM NaF, 20 μg/ml leupeptin, 1 mM phenylmethylsulfonfluoride, and protease inhibitor mixture and centrifuged at 20,442 × g for 15 min at 4 °C. Synapsin I, dynamin I, and synaptotagin were affinity-purified from the supernatant on bacterially expressed glutathione S-transferase (GST) fusion proteins containing the GST-Grb-2-SH3 domains bound to glutathione-Sepharose (GSH-Sepharose) were mixed with the supernatants for 1 h at 4 °C (19). The washed beads were heated in sample buffer and the released proteins separated by SDS-PAGE. In some experiments total homogenates from rat brain were sequentially extracted three times with GSH-Sepharose (to remove endogenous GST), followed by three extractions each with GSH-Sepharose-attached GST fusion proteins comprising the SH3 domains of Grb2, amphiphysin I or II, or p85. Each recombinant GST-SH3 domain was mixed with the tissue lysate after the preceding one was removed by filtration, in the order shown in the results.

MALDI-TOF MS—Proteins that bound to the GST-SH3 domains were separated from gels and submitted to in-gel tryptic digestion and analyzed by MALDI-TOF MS (20). Proteins were identified with the search engines Protein Prospector and Mascot, and a MOWSE score analyzed by MALDI-TOF MS (20). Proteins were identified with the search engines Protein Prospector and Mascot, and a MOWSE score analyzed by MALDI-TOF MS (20). Proteins were identified with the search engines Protein Prospector and Mascot, and a MOWSE score analyzed by MALDI-TOF MS (20).

RESULTS

Synapsin I and PI 3-Kinase Interact in Nerve Terminals—Synapsin associates with many SH3 domain-containing proteins, including Grb2, amphiphysins I and II, and p85 (11). To identify selective binding partners for PI 3-kinase in brain we performed sequential pull-down experiments using GST fusion proteins containing the SH3 domains of p85, Grb2, amphiphysin I (A1), amphiphysin II (A2) and identified interacting proteins by MALDI-TOF mass spectrometry. This sequential approach was aimed at pre-clearing strongly interacting partners like dynamin I, which may bind all the targets. Using GST-Grb2-SH3 first, the major protein extracted from brain homogenates was the highly abundant dynamin I (Fig. 1A). The homogenate was extracted twice more with Grb2-SH3 until no more dynamin I was recovered, then sequentially extracted with the other recombinant SH3 domains. When p85-SH3 extraction followed Grb2-SH3, synaptotagmin, and synapsins Ia and Ib were present (Fig. 1A). Subsequent extraction with A1-SH3 or A2-SH3 recovered more synaptotagmin and additional unique proteins (Fig. 1A). When the order of extraction was altered such that p85-SH3 was utilized last, an association with synapsins Ia and Ib was still observed, despite prior extraction with three other SH3 domains. Therefore the p85-SH3-synapsin association is highly selective when compared against other SH3 domain proteins and p85 may be a key synapsin-binding protein in nerve terminals.

To identify PI 3-kinase binding partners in nerve terminals, we performed pull-down experiments in synaptosomes using recombinant p85-SH3 or Grb2-SH3 (Fig. 1B). p85-SH3 primarily extracted synaptotagmin, dynamin I, and synapsin Ia/b. Grb2 also extracted synaptotagmin and dynamin I, but its association with synapsin was barely detectable, highlighting the relative selectivity of the p85-SH3-synapsin interaction. Protein identification was confirmed by MALDI-MS (not shown) and by Western blotting (Fig. 1C). To determine whether the phosphorylation status of the extracted proteins regulate their binding, pull-down experiments were performed in metabolically labeled, resting (Ctrl), depolarized (S1), repolarized (Rep), or redepolarized (S2) synaptosomes. No difference in the amount of protein bound was observed (Fig. 1B and data not shown) indicating these interactions are independent of their phosphorylation status (Fig. 1D).

To determine whether synapsin I and PI 3-kinase form a complex in intact nerve terminals, we performed a series of co-IPs. IP of p85 revealed synapsin I, as did IP of the catalytic subunit of PI 3-kinase, p110 (Fig. 2A). Conversely, synapsin antibodies immunoprecipitated p85. Synapsin and p85 also co-localize to SV clusters in specific puncta in the neurites of B104 neuronal cells differentiated with cAMP (Fig. 2, B and C).

Thus PI 3-kinase is found in the correct subcellular location for a role in SV recycling.

To determine whether synapsin associates with an active PI 3-kinase complex, we measured PI 3-kinase activity in synapsin I IPs (Fig. 2D). A high level of activity immunoprecipitated...
with synapsin 1 in resting nerve terminals. This activity increased about 2-fold after S1 and was reversed when the stimulus was removed (Fig. 2D, Repol). Thus synapsin and PI 3-kinase form an active complex in nerve terminals that is regulated by stimulation.

**PI 3-Kinase Regulates Glutamate Release from the Reserve Pool**—Next we examined the functional role of PI 3-kinase activity in neurotransmitter release from synaptosomes. Glutamate is the predominant neurotransmitter in synaptosomes, with greater than 80% of nerve terminals being glutamatergic (23). KCl-evoked Ca\(^{2+}\)-dependent glutamate release was strongly inhibited when synaptosomes were preincubated with either 1 μM Wortmannin or 100 μM LY294002 (Fig. 3A). Neither inhibitor had any effect on KCl-evoked Ca\(^{2+}\)-independent glutamate release (data not shown), ruling out effects on the plasma membrane glutamate co-transporter (23). The same results were obtained with 100 nM Wortmannin when preincubation times were increased to 60 min (data not shown).

Fast and slow phases of glutamate release have been characterized in synaptosomes and represent release from the RRP or RRP replenishment from reserve pool SVs, respectively (24, 25). Remarkably, Wortmannin or LY294002 exclusively inhibited the slow phase of Ca\(^{2+}\)-dependent glutamate release (Fig. 3A). We compared a number of inhibitors with different mechanisms of action for their relative effects on the two pools. We arbitrarily defined the release originating from the reserve pool as glutamate released after 90 s of stimulation, with glutamate release prior to 10 s being from the RRP and calculated a ratio (>90 s/10 s). A ratio of greater than one indicates preferential inhibition of the RRP, whereas a ratio less than one indicates a block of SV delivery from the reserve pool. The only antagonists that affected this ratio were Wortmannin and LY294002, which reduced the ratio value to 0.50 ± 0.02 and 0.36 ± 0.04, respectively (Fig. 3B). This indicates that PI 3-kinase activity may be required for the delivery of reserve pool SVs to the RRP during prolonged depolarization.

To ensure that the requirement for PI 3-kinase activity is upstream from the RRP, SVs from the RRP were selectively released by application of hypertonic sucrose (26). This protocol only releases docked and primed SVs and does not require Ca\(^{2+}\)-influx. Application of hypertonic sucrose in the presence or absence of extracellular Ca\(^{2+}\) resulted in the release of 2.5 ± 0.3 or 2.4 ± 0.2 nmol/mg glutamate, respectively, an amount expected for release exclusively from the RRP. When synaptosomes were preincubated with Wortmannin or LY294002, no inhibition of sucrose-evoked glutamate release was observed either in the presence or absence of extracellular Ca\(^{2+}\) (Fig. 3C). Therefore PI 3-kinase activity is not required for the fusion of SVs that are already present in the RRP.

The inhibition of a late phase of glutamate release from synaptosomes correlates with a block of SV delivery from the reserve pool. To determine whether a similar inhibition might occur during periods of high synaptic transmission in situ, we made whole-cell patch-clamp recordings of EPSCs from hippocampal CA1 pyramidal neurons after a prolonged train of repetitive stimulation. When a prolonged repetitive stimula-
Fig. 2. Synapsin and p85 form a complex in nerve terminals. A, a series of co-IPs reveal a protein complex between synapsin I and PI 3-kinase in nerve terminals. IPs with antibodies to p85 or p110 recovered synapsin I, whereas an IP of synapsin recovered p85. B, immunofluorescence in B104 neurites using synapsin (green) and p85 (red) antibodies. Yellow in the merged image shows co-localization at puncta of clustered SVs but no specific co-localization in the spaces between these clusters. C, co-localization of p85 and synapsin at contacts (long arrow) between neurites from separate B104 cells as well as at neurite branch points (short arrow). No specific co-localization occurred in the neurites other than the clusters of SVs. D, synapsin I IP from resting (Ctrl), depolarized (S1), or repolarized (Repol) synaptosomes were assayed for PI 3-kinase activity. Data are a percent of PI 3-kinase activity immunoprecipitated from resting synaptosomes, n = 4 ± S.E.

The PI 3-Kinase-Synapsin Interaction Is Required for SV Delivery—We next addressed whether the synapsin-PI 3-kinase interaction plays a role in the late phase of glutamate release. We designed a potential inhibitory peptide (SynI585–600) similar to one previously used to block the synapsin-p85 association in vitro (11). The peptide abolished the interaction of synapsin I with p85-SH3, but not that of dynamin I or synaptotagin (Fig. 4, A and B). This is important because dynamin I is a major ligand of the p85-SH3 domain in nerve terminals (Fig. 1B). Two additional synapsin I peptides that contain potential SH3-binding motifs were without effect (Fig. 4, A and B).

Peptides from the third α-helix of the homeodomain of Drosophila antennopedia (penetratin43–58) are used for translocating peptide into cells (30). To examine the consequences of disrupting the p85-synapsin I interaction in nerve terminals, the SynI585–600 peptide was translocated into synaptosomes using this vector system. We employed the minimum sequence required for internalization, the C-terminal heptapeptide (penetratin42–50) (17) (Fig. 4D). This approach complements pharmacological studies, because PI 3-kinase activity is not inhibited. The penetratin-SynI585–600 peptide abolished the p85-synapsin I interaction but importantly did not affect the dynamin I or synaptotagamin interactions (Fig. 4C).

The penetratin-SynI585–600 peptide inhibited only the late phase of Ca2+-dependent glutamate release (Fig. 5A). It did not affect Ca2+-independent glutamate release (data not shown), a reliable indicator that nerve terminal viability was not compromised. The selective inhibition of reserve pool glutamate release by penetratin-SynI585–600 was clearly indicated when exocytosis was separated into early (release <10 s, inhibition of 6.1 ± 12.4%, n = 4 ± S.E.) and late components (release >90 s, inhibition of 40.4 ± 3.8%, n = 4 ± S.E.). This is reflected in the reserve/RRP ratio (Fig. 5B). The peptide was without effect on RRP release evoked by hypertonic sucrose (Fig. 5C). To control for potential side-effects of the penetratin leader sequence, peptides identical to the clathrin-binding site on AP-2 (AP-2 β2624–644) (31) were prepared with the same leader sequence (Fig. 5F). AP-2 β2624–644 had no effect on glutamate release (Fig. 5D) and blocked endocytosis, as expected (Fig. 5E). Thus the data suggest that the direct interaction of PI 3-kinase with synapsin I is important for the delivery of SVs to the RRP but not for the fusion of SVs present in the RRP.

The first stage of SV delivery is liberation of SVs from the reserve pool (6). Because site-specific phosphorylation of synapsin I controls this process, PI 3-kinase may mediate liberation by altering synapsin phosphorylation, either directly or via a downstream protein kinase such as Akt (6). However, we
found that neither Wortmannin nor LY294002 had any effect on the depolarization-dependent phosphorylation of synapsin I in synaptosomes, ruling out this possibility (Fig. 6).

**DISCUSSION**

Our data reveal a new role for PI 3-kinase in the delivery of SVs from the reserve pool to the RRP for exocytosis. This role is mediated by an association between PI 3-kinase and synapsin I, via p85. This is the first report of a specific locus of action of PI 3-kinase activity in SV exocytosis. Our conclusions are based on a selective inhibition of the late phase of glutamate release by both PI 3-kinase inhibitors and a peptide that blocks the synapsin-p85 interaction. This inhibition is not due to a block in SV recycling because none of these treatments had any effect on SV endocytosis (not shown). The early and late phases of glutamate release are well characterized in synaptosomes and almost all treatments that reduce exocytosis do so equally from both phases (24, 25). A similar selective inhibition of KCl-
evoked glutamate release is also observed in synaptosomes derived from synapsin I knockout mice (5), demonstrating the synapsin-dependent nature of this late phase. Thus the interaction of PI 3-kinase with synapsin during this phase may be important for targeting PI 3-kinase to phosphorylate inositol lipids either on the SV or on the plasma membrane. The most likely explanation for our results is that production of PI(3,4,5)P₃ is necessary for the delivery of SVs to the RRP after their liberation from the reserve pool. SV delivery has two steps, translocation and entry into the RRP. Translocation from SV clusters in the reserve pool to the RRP may occur by passive diffusion, or mechanisms may exist to direct a subset of SVs to release sites. SV entry into the RRP is a two-stage process, mediated by SV docking at active zones followed by a priming event that confers fusion competence on the SV. It is unlikely that the synapsin-PI 3-kinase association plays a role in SV entry into the RRP as synapsin itself is not required for this step (6).

PI 3-kinase mediates the specificity of vesicle delivery at various stages of vesicle recycling pathways in other cells; however, different isoforms play distinct roles. Type III PI 3-kinase mainly produces PI (3)P and confers specificity of docking of vesicles at endosomes (32, 33). Type II PI 3-kinase plays a role in clathrin-coated bud formation at the plasma membrane and at the trans-Golgi surface via its association with clathrin (34). Type I PI 3-kinases, as described in this paper, produce PI(3,4,5)P₃ as their major product. }
through the actin cytoskeleton in a synapsin-independent manner (15, 16). PI 3-kinase plays key roles in regulation of the actin cytoskeleton (40–42). For example it directly associates with and activates p21-activated kinase-1, which is downstream of the small GTPases Rac and Cdc42 (43) and directly binds active Rac or Rho (44, 45). PI 3-kinase also interacts with profilin (46) (which also binds PI(3,4,5)P3), increasing the interaction of this complex with actin (47). In chromatin cells, PI 3-kinase inhibitors block exocytosis by specifically affecting actin assembly (40). Our results link these effects with the role of synapsin I in the actin cytoskeleton and provide a framework for how these proteins may work together at synapses. Thus synapsin serves as an anchor regulating the localization and activation of PI 3-kinase at SV clusters to regulate SV trafficking from the actin cytoskeleton. In support, p110-α is highly enriched in the growth cones and filopodia of cultured primary hippocampal neurons where it is coincident with enriched F-actin (48).

Our results indicate a new role for synapsin I-associated PI 3-kinase activity in glutamate release from central nerve terminals. PI 3-kinase inhibition also enhanced the rundown of synaptosomal glutamate release from central nerve terminals. PI 3-kinase inhibition also enhanced the rundown of synaptosomal glutamate release from central nerve terminals. PI 3-kinase inhibition also enhanced the rundown of synaptosomal glutamate release from central nerve terminals.
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J. Biol. Chem. 2003, 278:29065-29071.
doi: 10.1074/jbc.M302386200 originally published online May 16, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M302386200

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