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Calcineurin Selectively Docks with the Dynamin Ixb Splice Variant to Regulate Activity-dependent Bulk Endocytosis

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Depolarization of nerve terminals stimulates rapid dephosphorylation of two isoforms of dynamin I (dynI), mediated by the calcium-dependent phosphatase calcineurin (CaN). Dephosphorylation at the major phosphorylation sites Ser-774/778 promotes a dynI-syndapin I interaction for a specific mode of synaptic vesicle endocytosis called activity-dependent bulk endocytosis (ADBE). DynI has two main splice variants at its extreme C terminus, long or short (dynIxa and dynIxb) varying only by 20 (xa) or 7 (xb) residues. Recombinant GST fusion proteins of dynIxa and dynIxb proline-rich domains (PRDs) were used to pull down interacting proteins from rat brain nerve terminals. Both bound equally to syndapin, but dynIxb PRD exclusively bound to the catalytic subunit of CaNA, which recruited CaNB. Binding of CaN was increased in the presence of calcium and was accompanied by further recruitment of calmodulin. Point mutations showed that the entire C terminus of dynIxb is a CaN docking site related to a conserved CaN docking motif (PXIXI(T/S)). This sequence is unique to dynIxb among all other dynamin variants or genes. Peptide mimetics of the dynIxb tail blocked CaN binding in vitro and selectively inhibited depolarization-evoked dynI dephosphorylation in nerve terminals but not of other dephosphins. Therefore, docking to dynIxb is required for the regulation of both dynI splice variants, yet it does not regulate the phosphorylation cycle of other dephosphins. The peptide blocked ADBE, but not clathrin-mediated endocytosis of synaptic vesicles. Our results indicate that Ca2+- and calmodulin-dependent protein phosphatase calcineurin (CaN, or protein phosphatase 2B) upon nerve terminal depolarization (14−17). CaN is the only protein phosphatase directly regulated by Ca2+ and calmodulin (18−20). It is a heterodimer of a 60-kDa catalytic subunit, CaNA, and a 19-kDa regulatory subunit, CaNB. DynI interacts directly with CaN in a strictly Ca2+-dependent manner via its PRD (21, 22); however, no functional role has been identified for this interaction. In contrast, the activity-dependent dephosphorylation of dynI by CaN is essential for the triggering of ADBE. DynI dephosphorylation permits its association with syndapin I, a protein that is essential for ADBE but not CME (3, 23). Thus, CaN activity is required to trigger ADBE in central neurons to increase endocytic capacity under periods of unusually intense activity.

The dynamins are a superfamily of up to seven cellular GTPase enzymes, which each play a role in membrane fission or fusion (1). There are three “classical dynamins,” dynI, -II, and -III, which mediate the internal fission of vesicles from cell membranes. The neuronal isoform of dynamin, dynI, is essential for all forms of synaptic vesicle (SV) endocytosis in central nerve terminals (2, 3). At least three different SV endocytosis modes are proposed to exist in nerve terminals. The kiss-and-run mode has no defining biochemical or morphological markers, and thus its existence is still a matter of controversy (4). Clathrin-mediated endocytosis (CME) retrieves single SVs from the plasma membrane and is the dominant endocytosis mode during mild stimulation (5). In contrast, activity-dependent bulk endocytosis (ADBE) invaginates large areas of plasma membrane to form bulk endosomes (BEs) from which SVs can later bud and rejoin the recycling pool (6, 7). ADBE is only triggered during high intensity stimulation and is the dominant SV endocytosis mode under these conditions (8).

CME and ADBE appear to utilize similar or the same endocytic proteins (9, 10), with some exceptions (3, 11). In agreement, dynI GTPase activity is essential for both modes (3). However, ADBE is additionally reliant on the dephosphorylation and rephosphorylation of dynI (3, 12, 13). DynI is the founding member of a group of endocytic proteins called the dephosphins that are dephosphorylated by the Ca2+- and calmodulin-dependent protein phosphatase calcineurin (CaN, or protein phosphatase 2B) upon nerve terminal depolarization (14−17). CaN is the only protein phosphatase directly regulated by Ca2+ and calmodulin (18−20). It is a heterodimer of a 60-kDa catalytic subunit, CaNA, and a 19-kDa regulatory subunit, CaNB. DynI interacts directly with CaN in a strictly Ca2+-dependent manner via its PRD (21, 22); however, no functional role has been identified for this interaction. In contrast, the activity-dependent dephosphorylation of dynI by CaN is essential for the triggering of ADBE. DynI dephosphorylation permits its association with syndapin I, a protein that is essential for ADBE but not CME (3, 23). Thus, CaN activity is required to trigger ADBE in central neurons to increase endocytic capacity under periods of unusually intense activity.

DynI is a modular protein, containing an N-terminal GTPase module, middle, GTPase effector, pleckstrin homology domain, and proline-rich domain (PRD). The dynI PRD is located at the C terminus, which is the main site of phosphorylation and an interaction platform for a variety of Src homology 3 (SH3) domains from other proteins. Rat dynI has three sites for alternative splicing that produce up to eight mRNAs (UniProtKB accession number P21575) (24); however, there is limited understanding of the functional roles of these splice variants in neurons (25−27). The dynI third splice site produces three
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alternative C-terminal tails beginning at residue 845, dynIxa (long), dynIxb (short), or dynIxd (intermediate). The two major spliced tails (xa and xb) have an approximate ratio of 5:4 (xa/ (xb + xd)) in nerve terminals, with dynIxd expression being ~10% of the others (27). DynIxa has a unique 20-residue tail after Pro-844, 844PSRSGQASPSRPESPRPPFDL864, which includes several potential additional binding sites for SH3 domain-containing proteins. In contrast, the unique 7-residue tail of dynIxb, 844PRITISDP851, does not. Both dynIxa and dynIxb are phosphorylated and dephosphorylated upon electrical or chemical depolarization to approximately equal extents using metabolic $^{32}$P labeling, suggesting that they are coordinately regulated (28). However, quantitative mass spectrometry (which also detects unlabeled phosphorylation sites) revealed major differences in the distribution of phosphate between these sites in the dynIxa and dynIxb (27). Thus, the presence of alternative binding platforms and differential phosphorylation of key sites supports the hypothesis that these dynI splice variants may have unique functional roles in nerve terminals.

To date, the only reported differences between any of the endogenously expressed dynI isoforms is a differential phosphoregulation of dynIxa in synaptosomes (27, 29). DynI is phosphorylated at seven sites in nerve terminals, with the majority (69%) of metabolic labeling with $^{32}$P, occurring on Ser-774 and Ser-778 in a region of the PRD called the phospho-box (13, 29). However, two additional phosphosites in nerve terminals are unique for dynIxa, Ser-851 and Ser-857 (29). Among these two sites, Ser-857 is phosphorylated by Dyrk 1A/minibrain kinase in vitro, and this appears to affect amphiphysin I binding to this isoform (30, 31).

In an attempt to uncover different roles for different dynI splice variants in nerve terminals, we searched for protein partners whose interaction may be specific for either dynIxa or dynIxb. We report here that CaN uniquely associates with the short (xb) tail and not with the long (xa) tail of dynI. We characterized the dynIxb-CaN interaction, mapped the binding site, and found that the interaction was associated with a specific endocytic mode, ADBE. Our results support the model whereby dynI dephosphorylation by CaN is the primary trigger for ADBE, leading to the formation of a dynI-syndapin I complex as the next step in the process.

EXPERIMENTAL PROCEDURES

Materials—Leupeptin was from Merck. Tissue culture plastics were from Falcon (Franklin Lakes, NJ). Tetramethylrhodamine-dextran, penicillin/streptomycin, phosphate-buffered saline, minimal essential medium, and fetal calf serum were from Invitrogen. All other materials were from Sigma unless otherwise stated. Antibodies to PP2B-A (CaN-A), PP2B-B (CaN-B), amphiphysin I, and syndapin I were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); antibodies to dynI (hudy1) or calmodulin were from Millipore; and secondary antibodies were all from Dako. Three penetratin-based peptides, RMRKWKK-GVPRITISDP (short peptide), RRMKWKK-GVPRITISDP (mutant short peptide), and RRMKWKK-GVPRITISDP (long peptide), were synthesized by Auspep (Parkville, Australia). The pBJ5mCaNB (mouse, plasmid 17872), pBJ5-CaNA (mouse, plasmid 11785), and pGEX-CaNA(2–347) (human, plasmid 13251) plasmids were from Adgene Inc.

Pull-down Experiments and Mass Spectrometry—Crude (P2) synaptosomes were prepared from rat brains (25) and lysed in ice-cold buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 20 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and EDTA-free Complete-protease inhibitor tablets (Roche Applied Science). The homogenate was centrifuged at 75,600 g for 30 min at 4°C. The extract was precleared with GSH-Sepharose beads coated with GST recombinant proteins at 4°C for 1 h. The beads were isolated and washed extensively with ice-cold lysis buffer and eluted in 2× concentrated SDS sample buffer. Bound proteins were separated by 7.5–15% gradient SDS-PAGE and stained with colloidal Coomassie Blue. Protein bands were then excised from gels and identified by LC-MS/MS (QSTAR XL mass spectrometer, AB SCIEX) and searched against protein databases as described (27). See supplemental Table 1 for more details.

Synaptosomes and $^{32}$P Labeling—P2 synaptosomes were prepared from adult rat brains and labeled with 0.75 mCi/ml $^{32}$P, for 1 h at 37°C in a low calcium Krebs-like buffer (118.5 mM NaCl, 4.7 mM KCl, 1.18 mM MgCl2, 0.1 mM K2HPO4, 20 mM Hepes, 10 mM glucose, with 0.1 mM calcium, pH 7.4) and washed (25). In some experiments, the synaptosomes were depolarized by the addition of KCl to 41 mM for 10 s, were immediately lysed in ice cold lysis buffer (25 mM Tris, pH 7.4, containing 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 2 mM EDTA, 50 mM NaF, 20 μg/ml leupeptin, 1 mM PMSF, and EDTA-free protease inhibitor mixture), and were centrifuged at 20,442 g for 20 min at 4°C. Pull-down experiments were performed sequentially. First, the GST-amphiphysin 1-SH3 domain on GSH-Sepharose beads was used to pull down dynI and synaptotagmin from the lysate. Then GST-α-adaptin ear domain on GSH-Sepharose beads was used in a subsequent pull-down from the same lysate to isolate AP180 and...
Fluorescence Imaging of Dextran Uptake—The uptake of tetramethylrhodamine-dextran (40 kDa, 50 μm) was monitored as described previously (3) in cells stimulated with a train of 800 action potentials (delivered at 80 Hz, 1-ms pulse width) in incubation medium supplemented with horseradish peroxidase (HRP; 10 mg/ml) and peptide where indicated. This stimulation protocol activates both CME and ADBE (8). Neurons were then immediately fixed in a 2% solution of glutaraldehyde in PBS for 30 min at 37°C. After washing in 100 mM Tris, pH 7.4, cultures were exposed to 0.1% diaminobenzidine and 0.2% H2O2 in 100 mM Tris, pH 7.4. Upon development of color, cultures were washed with 100 mM Tris and then postfixed with 1% osmium tetroxide for 30 min. After washing, cultures were poststained with 2% uranyl acetate for 15 min and then dehydrated using an ethanol series and propylene oxide and then embedded using Durcupan. Samples were sectioned, mounted on grids, and viewed using an FEI (Hillsboro, OR) Tecnai 12 transmission electron microscope. Intracellular structures that were <100 nm in diameter were arbitrarily designated to be SVs, whereas larger structures were designated to be endosomes. All experiments were performed on cultures between 8 and 10 days in vitro. For transfection of CaNA or CaNB into mammalian cells, COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, and the transfection reagent was FuGENE 6 (Roche Applied Science).

RESULTS
Calcineurin Selectively Binds the Short DynIxβ Splice Variant—DynIxα and dynIxβ differ in the amino acid sequences of their alternatively spliced tails (Fig. 1A) (24, 25). To search for poten-
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tional isoform-selective binding partners for the two tails, both PRDs were expressed as GST fusion proteins bound to GSH-Sepharose beads and incubated with rat brain synaptosomal lysate for pull-down experiments. Proteins at about 58 and 19 kDa selectively associated with one form, dynlxb PRD (Fig. 1, B and C). The 58-kDa protein was unequivocally identified by LC-MS/MS sequencing as the catalytic subunit of the serine/threonine-protein phosphatase 2B (CaN), also known as CaNA, and the 19-kDa protein was identified as the regulatory subunit of calcineurin, CaNB. The 58-kDa protein had 19 matching peptides with 38% sequence coverage of CaNA α isoform 1 and 10 matching peptides with 36% sequence coverage of CaNA α isoform 2 (supplemental Table 1). It also had eight matching peptides with 20% sequence coverage of CaNA β isoform, three peptides being unique to the β not α isoform (supplemental Table 1). Therefore, both CaNA α and β forms were unequivocally detected. The 19-kDa protein revealed seven matching peptides with 53% sequence coverage of CaNB, matching both known isoforms 1 and 2 (supplemental Table 2). The identity of these proteins as CaNA and CaNB was independently confirmed by Western blotting with specific antibodies (Fig. 1D). The blot was stripped and reprobed with anti-amphiphysin I antibodies, and amphiphysin I was found to bind to xa and xb to about the same extent (Fig. 1D). The results show that both CaNA and CaNB specifically interact with the short dynlxb splice variant but do not yet reveal which is the primary contact.

We next explored the potential Ca$^{2+}$ dependence of this interaction. In the presence of 200 μM Ca$^{2+}$, both CaNA and CaNB increased binding to the dynlxb PRD (Fig. 1E and supplemental Fig. S1, A and B). Interestingly, an additional band at 21 kDa was also detected in the Coomassie-stained gel, binding specifically to dynlxb PRD (supplemental Fig. S1B). LC-MS/MS clearly identified six tryptic peptides with 50% sequence coverage of calmodulin (CaM) (supplemental Table 3). This was confirmed by immunoblot analysis with specific anti-CaM antibodies, which also revealed that CaM bound to the dynlxb-CaM complex only in the presence of Ca$^{2+}$ (Fig. 1E, bottom). Because CaNA and CaNB are known to be a complex, we asked which is the primary contact with dynlxb. We transfected CaNA or CaNB, or we cotransfected both, into COS7 cells. The cell lysates were incubated with GST-dynlxb PRD on GSH-Sepharose beads. Western blots showed that CaNA bound directly associated with dynlxb PRD (Fig. 1F, top), whereas CaNB did not bind alone, but bound dynlxb through CaNA (Fig. 1F, bottom). The amount of CaNA bound to the dynlxb PRD in the presence of various concentrations of Ca$^{2+}$ was further explored by Western blot, and the level of bound CaNA was quantified by densitometry. Maximal binding of CaNA to dynlxb PRD occurred at about 1 μM free Ca$^{2+}$, with an estimated EC$\textsubscript{50}$ for Ca$^{2+}$ of 0.1 μM (Fig. 1G). This result is consistent with the previously reported binding of dynl to CaN for calcium (EC$\textsubscript{50}$ in the range of 0.1–0.4 μM) (21)). This affinity was similar to the in vitro affinity of CaN for calcium ($K_v$ value is less than 1 μM) (35)). The results show that it is the catalytic subunit that interacts with dynlxb and that it has a high degree of constitutive binding.

Mapping the CaNA Binding Region in the Dynlxb Short Tail—To characterize dynlxb-CaNA binding, we used dynl PRD to pull down proteins from synaptosomal lysates. Note that there

![FIGURE 2. Mapping the CaN binding site within the dynlxb PRD. A, CaN specifically binds to dynlxb PRD but not dynlxa. Synaptosomal lysates were incubated with GST-dynl PRD (either xa or xb) or GST-dynl PRD coupled to GSH-Sepharose beads, and CaNA binding was revealed by Western blot with anti-CaNA antibodies. B, dynlxb specifically binds to GST-CaNA. Synaptosomal lysates were incubated with either GST-CaNA(2–347) or GST-AmphII-SH3 coupled to GSH-Sepharose beads. Bound proteins were stained with Coomassie Blue after separation by an acrylamide gel with low bis-acrylamide and elevated pH, which is known to resolve the dynl splice variants (27). C, CaN binds to the alternatively spliced C-terminal tail of dynlxb(844–851). Single point mutations were made in the PRITIS motif of dynlxb PRD. The bacterially expressed mutant GST-dynlxb PRDs were used for pull-down experiments from synaptosomal lysates. Bound proteins were subjected to Western blotting with anti-CaNA antibodies. D, mutations in the PRITIS motif do not affect amphiphysin I or syndapin binding to dynlxb PRD. The penetratin-tagged peptide mimetic of the short variant, dynlxb(844–851), containing the entire spliced insert was synthesized and used for competition studies. Syndapin binding to dynlxb PRD was analyzed by Western blot analysis with antibodies against CaNA, CaNB, syndapin, and amphiphysin I. F, mutant peptide competition determined the specificity of the binding of CaN to the C terminus of dynlxb PRD. The penetratin-tagged peptide containing ARATA was synthesized and used for competition studies. Synaptosomal lysates were incubated with GST-dynlxb PRD immobilized on GST-Sepharose beads in the absence or presence of 5–100 μM peptide. Binding of proteins was detected by Western blot analysis with antibodies against CaNA, CaNB, syndapin, and amphiphysin I. E, mutant peptide competition revealed binding of CaN to the C terminus of dynlxb PRD. The penetratin-tagged mutant peptide containing ARATA was synthesized and used for competition studies. Syndaposomal lysates were incubated with GST-dynlxb PRD immobilized on GST-Sepharose beads in the absence or presence of 5–100 μM mutant peptide. Binding of proteins was detected by Western blot analysis with antibodies against CaNA, syndapin, and amphiphysin I. G, CaN binding to GST-dynlxb PRD is unaffected by the alternatively spliced C-terminal tail of dynlxb(842–864). The penetratin-tagged peptide dynl(842–864) was synthesized and used for a pull-down experiment as in E. CaN binding to the dynlxb PRD was analyzed by Western blot with anti-CaNA antibodies. All results are representative of two independent experiments. AmphII and AmphIII, amphiphysin I and II, respectively.]

is only one known C-terminal tail for dynlI, and its amino acid sequence is more similar to that of dynlxa. No CaNA was detected by immunoblot on dynlI PRD (Fig. 2A). We also used GST–CaNA(2–347)-coated GSH-Sepharose beads to pull down its binding partners from synaptosomal lysate. The main Coomassie protein band found to bind GST–CaNA(2–347) comigrated on gels with the short form of dynlI (dynlxb) (Fig. 2B, note the lower band of the doublet), whereas in contrast,
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GST-Ampl-SH3 is known to associate with dynl splice variants, including dynixa, dynxb, and dynlxd (27). Mass spectrometry analysis of the main band confirmed that CaNA binds dynxb with considerable specificity (data not shown). Due to the sensitivity provided by mass spectrometry analysis, both dynixa (in the upper band) and dynlxd (in both upper and lower bands) were detected, but not dynlx. Considerably less Coomassie protein stain was detected for dynixa (Fig. 2B, lanes 1 and 2 versus lanes 3 and 4, upper band), despite the fact that dynamin family members are well known to dimerize with each other (36). It was not possible to quantify changes in dynlxd binding, but like dynixa, it has no specific sequence motif to indicate that it might interact with CaNA directly rather than indirectly via dynlx. These results suggest that CaNA specifically interacts with the 7-amino acid tail that is unique to dynlx.

Our next aim was to identify the binding site for CaNA in the short dynlx variant. Inspection of the dynlx tail sequence (PRITISDP) revealed a strong similarity to the conserved CaN docking motif PXIXI(T/S) (37, 38). To evaluate whether the “PRITIS” region of dynlx might be responsible for the CaN interaction, we performed single point mutations on each of the three amino acids conserved in the motif. GST-dynlx PRD mutated at any of the three sites (P844A, I846A, or I848A) failed to bind any CaN in pull-downs from nerve terminal lysates (Fig. 2, C and D). Binding to amphiphysin I and syndapin were not affected by these mutations (Fig. 2D). To confirm that CaNA specifically binds to the short dynlx tail, a synthetic PRITIS peptide containing dynlx(842–851) (GVPRITISDP) was fused with a short penetratin heptapeptide RRMKWKK (“short peptide”) and incubated with nerve terminal lysates prior to pull-downs with GST-dynlx PRD. The peptide reduced binding of CaNA, CaNB, and CaM to the dynlx PRD in a concentration-dependent manner, both in the absence (Fig. 2A) and in the presence of Ca$^{2+}$ (supplemental Fig. S2A), whereas the peptide did not affect syndapin and amphiphysin I binding to dynlx PRD (Fig. 2E). In contrast, a mutant PRITIS peptide, ARATASDP, fused with RRMKWKK, did not disrupt any of the CaNA-dynlx, syndapin-dynlx, and amphiphysin I-dynlx interactions (Fig. 2F).

Another peptide containing the long dynlx tail, dynlx(842–864) (RRMKWKK-GVPSRSQASPSRPESPRFDFL) (long peptide), also did not disrupt the CaN-dynlx interaction (Fig. 2G). Thus, we conclude that the PRITIS motif in the dynlx C-terminal splice site is the docking site for CaNA, via the parallel use of both mutagenesis and competitive peptides.

To determine whether CaNA binding might be regulated by dynl phosphorylation, we performed pull-down experiments with GST-dynlx PRD containing single or double point mutations that introduce pseudophosphorylation at Ser-774 and/or Ser-778, which are phosphosites known to regulate the interaction of dynl with syndapin I (23). These were without effect on CaNA binding (supplemental Fig. S2B). The results suggest that dynamin phosphorylation in the phospho-box region does not regulate CaNA binding to dynlx. To determine whether other protein-protein interactions might regulate CaNA binding, we prebound the GST-dynlx PRD with His-tagged full-length syndapin (which is its main binding partner for ADBE) and then performed the pull-down in synaptosomal lysates with this complex. Prebinding of syndapin abolished the subsequent CaNA-dynl interaction (supplemental Fig. S2C).

CaN Dephosphorylation of both Dynlxa and Dynlxb Splice Variants Requires CaN Binding to the Short Form—CaN dephosphorylates both dynlx and dynlxd to an approximately equal extent during nerve terminal depolarization (28). To determine whether dynlx dephosphorylation by CaN requires CaN binding to dynlx, we utilized $^{32}$P$^{-}$-labeled synaptosomes that were depolarized with KCl either in the absence or presence of the dynlx peptide or the CaN inhibitor cyclosporin A. The dynlx peptide and its inactive mutant control peptide were tagged with a penetratin heptapeptide to facilitate entry into synaptosomes (3, 39). Labeled synaptosomal lysates were subjected to a pull-down with GST-amphiphysin I-SH3 domains on GSH beads, which extracted dynl and synaptojanin. The lysates were then subjected to a second sequential pull-down with GST-α-adaptin ear domain to further isolate two other dephosphins, AP180 and amphiphysin I. Autoradiography revealed that cyclosporin A prevented CaN-mediated dephosphorylation of all four dephosphins in response to the KCl depolarization (Fig. 3A and supplemental Fig. S3, A and B; data shown in triplicate), but it did not affect the phosphorylation of the four proteins under the control conditions (supplemental Fig. S3, C and D). At a single concentration (100 μM), the dynlx peptide greatly reduced dynl dephosphorylation. Surprisingly, it affected the upper and lower bands of the dynl doublet to the same extent (Fig. 3A), despite the fact that CaN docking could be demonstrated only to the shorter xb form. When data from multiple independent experiments were pooled and quantified, it was revealed that both dynlxa and -xb dephosphorylation were significantly blocked by the xb peptide (Fig. 3, B and C). The effect was specific because the peptide did not prevent the dephosphorylation of the three other dephosphins examined (Fig. 3, D–F, and supplemental Fig. S3, A and B). In contrast, the inactive mutant peptide (100 μM) did not have any effects on any of the four proteins under any conditions (Fig. 3, A–C, and supplemental Fig. S3, A–D). Therefore, CaNA binding to a single dynamin splice variant regulates KCl-evoked dephosphorylation of two splice variants but not dephosphorylation of at least three other dephosphins.

The CaN-Dynlx Interaction Regulates ADBE but Not CME—The activity-dependent dephosphorylation of dynl by CaN and the subsequent interaction with syndapin I is specifically required for the ADBE mode of SV endocytosis, whereas dynl GTPase activity is required for both ADBE and CME (2, 3, 23). To determine whether the CaN-dynlx interaction is required for either SV endocytosis mode, the dynlx peptide (dynlx(842–851), GVPRITISDP) was introduced to primary cultures of cerebellar granule neurones, in which expression of dynlxa, -xb, and -xd was confirmed by pull-downs, Western blots, and mass spectrometry (supplemental Fig. S4, A and B). HRP was used as a fluid phase marker for parallel monitoring of both ADBE and CME because it has been extensively characterized in this neuronal system (3, 12, 40). ADBE is detected as the very rapid appearance of large BE structures (>100 nm in diameter) filled with electron-dense reaction product in nerve terminals, whereas CME is detected as electron-dense small SVs (<100 nm in diameter). Both ADBE and SVs were activated...
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A. Synaptosomes were metabolically labeled with 32P, washed, and incubated with or without 100 μM PRITIS or 100 μM ARATAS peptide or with 30 μM cyclosporin A for 15 min. Synaptosomes were then stimulated with 41 mM KCl for 10 s. After lysis, dynI and synaptojanin were isolated by pull-down with GST-amphiphysin I-SH3 bound to GSH-Sepharose beads, and dynIxa and -xb were separated by 12% acrylamide gel with 0.08% bisacrylamide and pH 9.2 (27). Proteins in the 100 kDa region containing the dynamins were visualized by Coomassie Blue staining to demonstrate even sample loading (top). Phosphorylated dynIxa and -xb were visualized by autoradiography of triplicate samples (bottom). Synaptojanin was visualized by a 7.5–15% gradient SDS-PAGE (supplemental Fig. S3). Following this pull-down, two additional dephosphins, AP180 and amphiphysin I, were extracted by a second sequential pull-down with GST-α-adaptin ear domain bound to GSH-Sepharose beads (Fig. 4, B–H). These results suggest that the interaction between dynIxb and CaN is required for ADPE but not for CME.

DISCUSSION

We have shown that a single neuronal splice variant of the classical GTPase dynI, dynIxb, possesses a selective docking site at its extreme C terminus for the catalytic subunit of the Ca2+-dependent protein phosphatase CaN. This docking site is unique to dynIxb and conforms to the consensus PXXLX(T/V) sequence observed in other CaN interaction domains. Inhibition of the CaN-dynIxb interaction resulted in a selective block of dynI dephosphorylation over other CaN substrates and a selective inhibition of ADPE over other SV endocytosis modes.

To independently confirm a block of ADPE, we used a complementary assay based on uptake of the large (40-kDa) fluorescent tetramethylrhodamine-dextran. This dextran selectively labels ADPE when applied during intense stimulation because it is too large to enter single SVs. In agreement with the HRP experiment, dextran uptake into nerve terminals does not occur during mild neuronal activity when only CME is active (8). The addition of the dynIxb peptide (60 μM) significantly reduced the uptake of dextran evoked by a train of 800 action potentials (80 Hz) when compared with control conditions, whereas the inactive mutant peptide did not have any effect (Fig. 4, F–H). These results suggest that the interaction between dynIxb and CaN is required for ADPE but not for CME.
Mammalian CaN does not bind to the vast majority of its substrates. However, it does bind to a selective small group of four, NFAT (45), the two-pore domain K+ channel TRESK (42), AKAP79 (46), and neutral sphingomyelinase 2 (47). They share in common a short consensus docking sequence motif, PXXI(T/S). This was first found in the sequence SPRIEIT in the NFAT family of proteins (37, 38) and subsequently in the others (42, 46–48). We found that GST-CaNA(2–347) pulled down mainly the short form of dynI from rat synaptosomal lysate despite the fact that it has been reported that different dynI isoforms can assemble with each other to form dimers or tetramers (36). In that study, more than 30–40% of one dynamin isoform could be co-immunoprecipitated by the antibodies against another isoform (36). However, our results show a surprisingly specific interaction of dynIxb with CaNA, and this raises the possibility that in nerve terminals, the short form might not necessarily occur as a multimer with the long form. This raises the possibility that there might be different pools of dynamin in neurons, some of which may contain just one single isoform, and is consistent with markedly different stoichiometry of phosphorylation of dynIxa and -xb in verve terminals (29).

We defined the CaN binding site in dynIxb by site-directed mutagenesis and peptide competition as being PRITIS, a sequence that conforms to the original CaN docking sequence SPRIEIT in NFAT. Docking of CaN to a select group of its substrates appears to be a conserved model to provide strong specificity to CaN signaling, despite the apparent availability of a much larger substrate selection. For example, the transcriptional activity of NFAT is dependent on its dephosphorylation

FIGURE 4. The CaN-dynIxb interaction is essential for ADBE. Cerebellar granule neuron cultures were incubated with 10 μg/ml HRP, and loading was stimulated by a train of 800 action potentials delivered at 80 Hz. The cultures were incubated with either wild-type (xb) or mutant containing ARATA (xb mut) penetratin-tagged peptide dynIxb(842–851) (60 μM) for 15 min before and during stimulation. A–C, HRP-labeled structures in typical fields of view either in the absence of peptide (A, Ctrl) or in the presence of mutant (B) or wild-type peptide (C). Scale bar, 150 nm in all images. D, mean number of HRP-labeled SVs generated per nerve terminal in the presence of the peptide. E, mean number of HRP-labeled endosomes generated per nerve terminal in the presence of the peptide. Data were pooled from either four (Ctrl) or three (xb mut and xb) independent experiments (Ctrl, n = 173 nerve terminals; xb mut, n = 119; xb, n = 87; all mean ± S.E.; ***, p < 0.001 compared with control, one-way analysis of variance; ns, not significantly different). F and G, dextran loading in typical fields of view in cerebellar granule neurons either in the absence of xb peptide (F) or in its presence (G). Scale bars (F and G), 15 μm. H, granule neurons were incubated with 50 μM tetramethylrhodamine-dextran and loading was stimulated by a train of 800 action potentials (80 Hz) followed by immediate dextran washout. Where indicated, cultures were incubated with 60 μM either wild-type or mutant xb peptide 15 min before and during stimulation. The data shown are mean number of dextran puncta per field of view as a percentage of control (n = 4 ± S.E.; ***, p < 0.01, one-way analysis of variance).
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by CaN (49–51). Disruption of the CaN-NFAT1 interaction with a SPRIEIT peptide inhibited NFAT dephosphorylation both in vitro and in vivo and also inhibited its nuclear translocation and gene expression in T-cells, without inhibiting the ability of CaN to dephosphorylate other endogenous proteins (37). Similarly, we discovered that the peptide containing the dynIxb(842–851) PIXIXIS motif prevented CaN interaction with dynIxb in vitro and selectively inhibited KCl-evoked dynl dephosphorylation in nerve terminals but not that of three other well characterized dephosphins, amphiphysin I, AP180, and synaptojanin. This demonstrates that the peptide did not block CaN activity but only its recruitment to dynIxb. Thus, the specific anchoring of CaN to dynIxb may be employed in the nerve terminal for ADBE in a similar manner to that seen with NFAT1 in the cell body for transcriptional regulation.

CaN dephosphorylates dynI only during periods of high activity. This event triggers an interaction with syndapin I for ADBE (3, 52). Our finding that the dynI tail PRITIS peptide dynIxb(842–851) selectively inhibits both ADBE and dynI phosphorylation adds considerable support to the model in which the action of CaN in ADBE is primarily mediated by dynI dephosphorylation. It may initially appear contradictory that phosphorylation adds considerable support to the model in which the action of CaN in ADBE is primarily mediated by dynI dephosphorylation. It may initially appear contradictory that dynI is selectively involved with only one mode of SV endocytosis when the use of the dynamin GTPase inhibitor dynasore has shown that dynI is essential for all modes (2, 3). However, there is a large pool of non-phosphorylated dynI in nerve terminals representing 80% of the total (27) that is not regulated by the signaling cascades that regulate ADBE (6, 13). Therefore, we propose that multiple pools of dynI reside in nerve terminals and that CaN binding and phosphorylation mechanistically separate a phospho-pool for ADBE, whereas the bulk of residual dynI is potentially available for CME or other functions. The ADBE-triggering action could therefore occur in two steps: first, the Ca2+-dependent recruitment of CaN to dynIxb (this study) and, second, the dephosphorylation of dynI, thereby triggering its binding to syndapin I (3, 13). Our results show that prior binding of syndapin to dynIxb prevents CaN recruitment. Because phosphorylation of dynI regulates its interaction with syndapin but not CaN, we hypothesize that the wild type dynI dissociates from CaN to associate with syndapin to play a key role in ADBE. Interestingly, both splice variants are dephosphorylated by CaN, suggesting that ADBE might utilize either variant.

Our results provide further evidence that CaN is required for ADBE via its interaction with dynIxb and dephosphorylation of dynI. In agreement, previous studies in chromaffin cells demonstrate a CaN-sensitive form of endocytosis that was only observed during high activity (53, 54). However, there are multiple CaN substrates in nerve terminals (e.g. the dephosphins) that are dephosphorylated independently of the CaN-dynIxb interaction and are linked to other SV endocytosis or trafficking modes (17, 55). This suggests that CaN activity may play other yet unidentified roles in the SV life cycle. Recent studies have raised the possibility of new functions for CaN in SV turnover, both during SV retrieval and during SV trafficking between intracellular pools (56, 57). It will be interesting to determine whether the targeted dephosphorylation of specific dephosphins may be responsible for these potential additional functions. How such targeting occurs remains to be determined, but possibilities include differential dephosphorylation by specific trains of neuronal activity, anchoring in different nerve terminal subdomains, or differential association with CaN-inhibitory proteins (58).

In summary, we found that a short alternatively spliced insert in dynnl confers the ability of a single splice variant to bind to an additional distinct partner, in addition to shared common binding partners like syndapin, endophilin, and amphiphysin. The CaN-dynIxb interaction, like the dynI-syndapin I interaction, is required for ADBE but not for CME in neurons. Our results suggest that CaN is specifically recruited to sites of ADBE by a selective docking to the tail of a single dynI splice variant. To the best of our knowledge, this is the first report of a selective cellular function for one of the dynI splice variants. Our results support a model whereby dynI dephosphorylation by CaN is the primary trigger for ADBE during intense neuronal stimulation, leading to the formation of a dynI-syndapin I complex as the next step in the process of bulk endocytosis.

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