Hippocalcin Functions as a Calcium Sensor in Hippocampal LTD

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Summary

It is not fully understood how NMDAR-dependent LTD causes Ca²⁺-dependent endocytosis of AMPARs. Here we show that the neuronal Ca²⁺ sensor hippocalcin binds the β2-adaptin subunit of the AP2 adaptor complex and that along with GluR2 these coimmunoprecipitate in a Ca²⁺-sensitive manner. Infusion of a truncated mutant of hippocalcin (HIP₂-72) that lacks the Ca²⁺ binding domains prevents synaptically evoked LTD but has no effect on LTP. These data indicate that the AP2-hippocalcin complex acts as a Ca²⁺ sensor that couples NMDAR-dependent activation to regulated endocytosis of AMPARs during LTD.

Introduction

α-aminoadamantane-5-ethyl-4-isoxazole propionic acid receptors (AMPARs) mediate most fast excitatory neurotransmission in the brain and play a central role in synaptic plasticity (Palmer et al., 2005). The postsynaptic expression of AMPARs is highly dynamic since they undergo both rapid constitutive (Nishimune et al., 1998) and activity-dependent endo- and exocytosis (Malinow and Malenka, 2002). During the induction of long-term depression (LTD), the synaptic activation of NMDARs drives endocytosis of AMPARs (Luthi et al., 1999; Carroll et al., 1999; Man et al., 2000) via a mechanism requiring the association of the AP2 heterotetramer with GluR2 (Lee et al., 2002). A fundamental unanswered question is how NMDAR activation triggers this process.

Hippocalcin is a high-affinity calcium-binding protein restricted to the CNS, most abundant in pyramidal cells of the hippocampal CA1 region (Kobayashi et al., 1993a, 1993b; Saitoh et al., 1993). It belongs to the family of EF-hand-containing neuronal calcium sensor (NCS) proteins that possess a Ca²⁺/myristoyl switch allowing translocation to membranes in response to increased cytosolic [Ca²⁺]. The role of hippocalcin is not yet clear, although it can regulate mixed lineage kinase 2 (MLK2; Nagata et al., 1998), phospholipase D (Hyun et al., 2000), and the neuronal apoptosis inhibitory protein (Mercer et al., 2000).

Here we have tested the hypothesis that hippocalcin may be a Ca²⁺ sensor in activity-dependent regulation of AMPAR endocytosis. We show that hippocalcin binds directly to the β2-adaptin subunit of the AP2 adaptor complex that couples clathrin to the cytosolic domains of integral membrane proteins destined to be internalized via the clathrin coat complex (McMahon and Mills, 2004; Owen et al., 2004). In neurons this hippocalcin-AP2 complex binds TIR in a Ca²⁺-independent manner, whereas the complex only binds to AMPARs in the presence of Ca²⁺. Infusion into CA1 pyramidal neurons of a truncation mutant of hippocalcin (GST-HIP₂-72) lacking the Ca²⁺-sensing motifs blocks synaptically evoked LTD without affecting basal AMPAR-mediated transmission or LTP. Furthermore, GST-HIP₂-72 does not inhibit constitutive TIR internalization in HeLa cells. These data indicate that (1) hippocalcin may be involved in general neuronal endocytosis and (2) that it plays a critical Ca²⁺-sensing role in NMDAR-mediated hippocampal LTD, where it couples NMDAR activation to the endocytosis of AMPARs.

Results and Discussion

Hippocalcin Binds AP2

Using full-length hippocalcin as bait in the yeast two-hybrid assay, we isolated a partial clone encoding 742 amino acids that represents ~70% of the N-terminal region of the β2-adaptin subunit of AP2 (truncated β2-adaptin, Figure 1A). To test the specificity of the interaction, the AP2 subunit “fish” clone was expressed with a wide range of other “bait” clones. No positive AP2 interactions were detected in yeast cells expressing the C termini of AMPA (GluR1-4), kainate (GluR5-2a/b/c and GluR6), and NMDA (NR1, NR2A, NR1C) receptor subunits, the C termini of metabotropic glutamate receptors (mGluR1-8), the GABAβ receptor subunits (R1, R2), or calmodulin (data not shown).

To determine the site of AP2 binding on hippocalcin, we engineered three overlapping hippocalcin truncations (Figure 1A) and tested for interaction with the full-length β2-adaptin subunit of AP2 in the yeast two-hybrid assay. Only the truncation encoding the N-terminal region (amino acid residues 2–72 [HIP₂-72]) yielded a positive interaction. HIP₂-72 also interacts with the...
Figure 1. Hippocalcin Interacts with the N Terminus of the AP2 Subunit β2-Adaptin

(A) Hippocalcin is 193 residues long and contains an N-terminal myristoylation site and four EF-hand calcium-binding motifs. Full-length hippocalcin interacted with a clone consisting of the N-terminal region of the AP2 subunit β2-adaptin in the yeast two-hybrid. The N-terminal 72 amino acid residues of hippocalcin comprise the minimum interaction domain (HIP2-72), interacting with full-length β2-adaptin.

(B) A177 and A172 anti-hippocalcin antibodies immunoprecipitate a single protein at the expected molecular weight for hippocalcin from rat forebrain as detected by the reciprocal anti-hippocalcin antibody in Western blots. Immunoprecipitation is prevented by the inclusion of the immunizing peptide or the replacement of antibody with preimmune serum.

(C) Anti-hippocalcin antibodies recognize a single specific band from rat forebrain extract.

(D) Ca\(^{2+}\) independence of TfR coimmunoprecipitation from rat forebrain with A177 anti-hippocalcin antibody. Due to the intensity of the band, the TfR input was detected separately. A177 immunization peptide reduced binding, demonstrating the specificity of the coimmunoprecipitation.

(E) Ca\(^{2+}\) dependence of AP2 and GluR2/3 coimmunoprecipitation from rat forebrain with A177 anti-hippocalcin antibody. The presence of
originally isolated truncated AP2 subunit. The fact that full-length hippocalcin did not interact with full-length β2-adaptin was initially surprising, but we and others have obtained similar results for other validated protein interactors in the yeast two-hybrid assay (e.g., PICK1/PKCζ; Staudinger et al., 1995). It is likely that this is due to conformational restraints and/or steric hindrance occurring in the yeast nucleus.

We next raised novel polyclonal anti-hippocalcin antibodies (designated A177 and A172) against a peptide comprising the C-terminal nine amino acids. An identical sequence is present in VILIP-3, but this protein is only expressed in the cerebellum (Kajimoto et al., 1993). The specificity of purified A177 and A172 was verified by Western blots against myc-tagged hippocalcin expressed in COS-7 cells (data not shown). Both antibodies recognized a specific protein at the correct molecular weight from transfected cells and forebrain homogenate. Preimmune serum did not give any immuno-reactive bands, and inclusion of the peptide antigen prevented immunolabeling of A177 immunoprecipitates with A172 and vice versa (Figures 1B and 1C).

Ca**2+**-Dependent AMPAR Coimmunoprecipitation with Hippocalcin

A crucial test of the hypothesis that hippocalcin is involved in AMPAR endocytosis via an interaction with AP2 in brain is the coimmunoprecipitation of a protein complex. We therefore probed the anti-hippocalcin antibody immunoprecipitates with anti-GluR2/3 using anti-transferrin receptor (TfR) antibodies as a control. Antibody immunoprecipitates with anti-GluR2/3 using anti-transferrin receptor antibodies (designated A172 and A177 in the presence or absence of calcium (Figure 1D), consistent with the constitutive Ca**2+**-insensitive internalization of TfR (Warren et al., 1998). In contrast, GluR2/3 was only bound in the presence of Ca**2+** (Figures 1D and 1E). Inclusion of GST-HIP2-72 (Figure 1A) prevented the Ca**2+**-dependent coimmunoprecipitation of GluR2 and AP2. This indicates that the construct competes with endogenous hippocalcin for AP2 binding (Figure 1F).

Both CPG2 and Myo6 have recently been implicated in NMDAR-dependent internalization of AMPAR, but the effect of Ca**2+** on these proteins was not reported (Cottrell et al., 2004; Osterweil et al., 2005). Clathrin has been identified as a binding partner for another NCS protein, neurocalcin, but it is unclear whether that interaction is direct or requires other proteins in the complex (Livings et al., 2002). Since there is no direct interaction between hippocalcin and GluR2/3, these data indicate that hippocalcin complexes with AMPARs via its interaction with AP2.

We did not detect NR1 on reprobing the blots of the coimmunoprecipitates (data not shown). This suggests that either the hippocalcin-AP2 pathway is not used for NMDAR internalization or that there are only low levels of NMDAR endocytosis. There is good evidence for AP2 and clathrin-mediated endocytosis of NMDARs (reviewed by Nong et al., 2004), but the developmental profiles, triggers, interacting proteins, and dynamics are certainly different to those for AMPARs. Furthermore, it has been suggested recently that calpain degradation rather than dynamin/clathrin-mediated endocytosis could be responsible for the downregulation of functional NMDARs in cortical neurons (Wu et al., 2005).

The AP2 recognition motif on GluR2 is the basic residue sequence KRKM (Lee et al., 2002). A similar AP2 binding motif (KRLK) has been reported for synaptotagmin (Chapman et al., 1998; Haucke et al., 2000). Significantly, the AP2 binding site of synaptotagmin is not an internalization signal but, consistent with the mechanism proposed here for hippocalcin in NMDAR-evoked AMPAR endocytosis, it acts in combination with separate Ca**2+**-binding regions as a regulator of endocytosis (Jarousse and Kelly, 2001).

Cellular Localization of Hippocalcin and AP2

In neurons, strong hippocalcin immunoreactivity was detected throughout the cell body (excluding the nucleus) and processes (Figure 1G), with discrete areas of punctate labeling in the dendrites. Overlapping immunostaining was observed between hippocalcin and AP2 and between hippocalcin and GluR2 (Figure 1H). Hippocalcin was not present in glial cells (data not shown).

Developmental Profile of Hippocalcin, AP2, and GluR2/3 in Rat Brain Extracts

For the AP2-hippocalcin-AMPAR complex to be of physiological relevance, each of the proteins involved must be available in the same cellular compartments and expressed at overlapping developmental time points. We therefore assessed expression in rat brain (minus cerebellum) of each of the proteins at a series of age points. Hippocalcin expression was low at postnatal day 2 (P2) and P4, but demonstrated a marked upregulation at P6–P14. Levels remained stable from P14 to adult (Figure 2A). AP2 was present at relatively constant levels at all age points tested, whereas GluR2/3 was less abundant at P2, but reached a stable level by P4.

Effects of Ca**2+** on Compartmentalization of Hippocalcin

To determine the cellular localization of hippocalcin, we prepared a series of subcellular fractions (Figures 2B–
Figure 2. Developmental and Subcellular Profiles of Hippocalcin, AP2, and GluR2

(A) Hippocalcin expression in rat forebrain is developmentally regulated.
(B) Hippocalcin and AP2 have overlapping subcellular distributions in crude cell fractions. GluR2 is enriched, and hippocalcin and AP2 are present in the synaptosomal (Syn) fraction.
(C–E) Presence of hippocalcin (n = 3), AP2 (n = 4), and GluR2 (n = 4) in progressively more stringent detergent extractions of the postsynaptic density in extraction buffer control (C), plus 2 mM EGTA (E), or plus 2 mM Ca\(^{2+}\) (Ca). Graphs show results of densitometry normalized to the PSDI Ca\(^{2+}\) condition.
(F) Presence of proteins in purified rat brain clathrin-coated vesicles (CCVs).

Expression in Purified Clathrin-Coated Vesicles

To test if hippocalcin is present in clathrin-coated vesicles (CCVs), we purified CCVs from rat brain (Korolchuk and Banting, 2002) (Figure 2F). AP2 and clathrin were highly enriched in the CCV fraction, and GluR1 and GluR2 AMPAR subunits were present but not enriched. As expected, TIR was also present in the CCV fraction. Interestingly, however, hippocalcin was absent from CCVs. These results suggest that the interaction between hippocalcin and the AP2-AMPAR complex is transient and may only occur at the plasma membrane.

One possibility is that Ca\(^{2+}\)-activated hippocalcin recruits AP2 to the AMPAR receptor to initiate activity-dependent internalization and dissociates from the AMPAR-AP2 complex once it is incorporated into clathrin-coated vesicles. This scheme differs from the constitutively endocytosed TIR where no Ca\(^{2+}\)-activation step is required for complex assembly. AP2 has two well-characterized clathrin-binding motifs (Boehm and Bonifacino, 2001). One of these is present in the truncated form of \(\beta\)-2-adaptin that we isolated as a hippocalcin interactor, and an overlapping binding site for clathrin/hippocalcin in the C-terminal domain may provide the mechanism for hippocalcin dissociation as clathrin is recruited. This scheme is similar to that shown previously for the clathrin-mediated endocytosis regulatory protein eps15, which uncouples from AP2 during clathrin coat formation (Cupers et al., 1997).
GST-HIP<sub>2-72</sub> Blocks LTD

It has been reported previously that NMDAR-evoked AMPAR internalization requires association of AP2 (Lee et al., 2002). To assess the role of hippocalcin in LTD, we used the hippocalcin truncation fusion protein GST-HIP<sub>2-72</sub> that competes for AP2 and consequently GluR2 binding in coimmunoprecipitations (Figure 1F). Because HIP<sub>2-72</sub> lacks the Ca<sup>2+</sup>-binding domains required for activation, it should act as an inhibitor of endogenous hippocalcin activity.

We introduced GST-HIP<sub>2-72</sub> or, in interleaved controls, GST alone into CA1 pyramidal cells via a patch pipette. The proteins were infused into neurons for at least 30 min, and the effects on basal AMPAR-mediated EPSCs and synaptic plasticity were monitored. Neither GST-HIP<sub>2-72</sub> nor GST alone had any effect on EPSC amplitude during basal synaptic transmission (Figures 3A and 3B). Robust homosynaptic LTD was induced in control experiments with GST alone when an LTD pairing protocol was applied (LTD path: EPSC amplitude = 72% ± 5% of baseline, p < 0.001, n = 8; LTD path significantly different from control path, p < 0.005, n = 8; Figure 3C). However, in the presence of GST-HIP<sub>2-72</sub>, LTD was blocked (LTD path: EPSC amplitude = 90% ± 5% of baseline, p = 0.1, n = 9, LTD path not different from control path, p = 1.0, n = 9; Figure 3D). In contrast, LTP could be readily induced in neurons perfused with GST-HIP<sub>2-72</sub> of a similar magnitude to that induced in interleaved control experiments in neurons containing GST alone (GST-HIP<sub>2-72</sub>; LTP path EPSC amplitude = 157% ± 15% of baseline, n = 6; GST control, LTP path EPSC amplitude = 160% ± 14% of baseline, n = 8; LTP with GST-HIP<sub>2-72</sub> not different from LTP in GST control, p = 0.93; Figures 3E and 3F). Therefore, disruption of hippocalcin function specifically affects LTD.

GFP-HIP<sub>2-72</sub> Does Not Block TfR Uptake in HeLa Cells

To determine if HIP<sub>2-72</sub> acts selectively for AMPARs or has a general role in membrane protein internalization by sequestering AP2, we compared the effects of dominant-negative AP180 (dnAP180) and GFP-HIP<sub>2-72</sub> on Tf-Alexa 594 uptake in HeLa cells. Constitutive Tf-Alexa 594 internalization is inhibited in cells expressing dnAP180, but not in cells expressing GFP-HIP<sub>2-72</sub>, where uptake is unchanged from levels observed in nontransfected cells (see Figure S1 in the Supplemental Data available online).

These results provide evidence for a mechanism involving protein-protein interactions linking a Ca<sup>2+</sup> sensor to AMPAR internalization during synaptic plasticity. Our results complement the observation that infusion of a peptide that blocks the GluR2-AP2 interaction similarly prevented LFS-induced LTD but not the constitutive recycling of AMPARs (Lee et al., 2002). As illustrated in Figure 4, the simplest explanation is that hippocalcin is the Ca<sup>2+</sup> sensor that directs AP2 to AMPARs to enable their regulated internalization in response to appropriate synaptic activity. In this regard, the sustained but moderate increase in free Ca<sup>2+</sup> concentration required for LTD induction (Mizuno et al., 2001) and the Ca<sup>2+</sup>-binding profile of hippocalcin are
AMPAR internalization occurs (C).

Clathrin displaces hippocalcin prior to endocytosis, after which binds to the GluR2 subunit of AMPARs and recruits clathrin (B). and stimulates binding to

Evoked AMPAR Internalization

Figure 4. Schematic of the Role of Hippocalcin in Synaptically

and calcineurin, which have been implicated in LTD

but not constitutive, GluR2 internalization and thereby calcium, is a key component of NMDAR-dependent, as a sensor of the NMDAR-evoked rise in intracellular calcium. It colocalizes completely with β2-adaptin (Boehm and Bonifacino, 2001). Images were obtained with a Leica TCS-NT confocal laser-scanning microscope and analyzed using Adobe Photoshop.

Subcellular Fractionation

Subcellular fractionations were obtained by differential centrifugation (Gray and Whittaker, 1962). The PSD fractions were prepared as described previously (Carlin et al., 1980), with the following modifications. The synaptosome fraction was solubilized in ice-cold 0.5% Triton X-100 for 15 min and then divided into three. EGTA or CaCl₂ was added to a third of the synaptosomes to a final concentration of 2 mM, and the final third was left untreated. The synaptosomes were centrifuged at 32,000 × g for 20 min to obtain the PSD I pellet. PSD II and PSD III pellets were obtained by resuspending the PSD I pellet in 0.5% Triton X-100 and ice-cold 3% Sarkosyl (with the addition of either 2 mM EGTA or 2 mM CaCl₂ where appropriate), respectively. After 10 min incubation on ice, the insoluble fractions were separated by 1 hr centrifugation at 201,800 × g. All pellets were resuspended in either PBS or 40 mM Tris-HCl (pH 8.0).

Protein concentrations were determined using the BCA protein assay (Pierce), and equivalent protein concentrations were Western blotted. Western blots were analyzed using densitometry in the ImageJ package (www.nih.gov).

Preparation of Fusion Proteins

HIP₂-72 was cloned into pGEX-4T1 (Amersham Pharmacia Biotech) or pGFP-N1 (Clontech). Extracts of E. coli strain BL21 DE3 (Novagen) expressing GST and GST-HIP₂-72 were prepared using BugBuster (Novagen) in the presence of EDTA-free protease inhibitors (Roche). Supernatants were purified on glutathione Sepharose 4B (Amersham Pharmacia Biotech), eluted, and dialysed against phosphate-buffered saline for immunoprecipitation or 5 mM NaHPO₄, 0.9 mM KHPO₄, pH 7.2, for electrophysiology. The integrity of GFP-HIP₂-72 was confirmed by sequencing and with anti-GFP immunoblots.

Immunoblotting

Primary antibodies used were 1:500 anti-β2-adaptin (Santa Cruz), 1:1000 anti-α2-adaptin (Sigma), 1:1500 A177, 1:1000 A172 anti-hippocalcin (GlaxoSmithKline), 1:500 anti-GluR2, and 1:1000 anti-GluR2/3 (Chemicon). Immunoreactive bands were detected with HRP-conjugated anti-goat, -rabbit, or -mouse secondary antibodies (Sigma) and visualized using enhanced chemiluminescence (ECL).

Primary Hippocampal Cultures

Primary hippocampal cultures were prepared from embryonic day 18 rats as previously described (Perestenko et al., 2003).

Immunocytochemistry

Neurons were fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) and permeabilized with 0.1% Triton X-100. The cultures were incubated with 3% bovine serum albumin to block non-specific staining, and all incubations with antibodies were performed in 3% bovine serum albumin. Antibodies used and their dilutions were as follows. Primary antibodies: A172 anti-hippocalcin (GSK) 1:10, α2-adaptin (Sigma) 1:10, GluR2 (Chemicon) 1:10; secondary antibodies: 1:200, donkey anti-rabbit Texas red (Jackson Immunoresearch) and goat anti-mouse Oregon green (Molecular Probes). An antibody to α2-adaptin was sometimes used since it colocalizes completely with β2-adaptin (Boehm and Bonifacino, 2001).

Experimental Procedures

Yeast Two-Hybrid Assay

Full-length hippocalcin was subcloned into the pBTM 117 ADE yeast two-hybrid bait vector and used to screen an adult rat brain cDNA library (Clontech) in S. cerevisiae L-40 strain as described previously (Nishimune et al., 1996, 1998). Truncations comprising amino acids 2–72 (HIP₂-72), 65–175 (EF), and 168–193 (C-term) of hippocalcin were subcloned into pBTM117 ADE and tested for an interaction with full-length β2-adaptin (gift from T. Kirchhausen) subcloned into pGAD 10 (Clontech).

Anti-Hippocalcin Antibody Production

The peptide CDPSASQF (VA/7086) was purchased from Cambridge Research Biochemicals (Cleveland, UK) and used to immunize rabbits. Antibodies (A172 and A177) were affinity purified against SulfoLink gel (Pierce) coupled to the immunization peptide and then dialyzed against PBS. 0.2% BSA was added to stabilize.

Immunoprecipitation from Rat Brain

Whole brains minus cerebellum were homogenized in a glass-teflon homogenizer in 10 volumes of tysis buffer (4 mM HEPES, 1% Triton X-100, protease inhibitor cocktail-EDTA [Roche], pH7.4) and spun at 10,000 × g. Supernatant was used in subsequent experiments. 7.5 μg/ml A177/A172 anti-hippocalcin or 20 μl preimmune serum was incubated with 5 mg brain extract, with or without 25 μg immunization peptide, 2 mM EDTA or CaCl₂ in NTN (20 mM Tris HCl, 100 mM NaCl, 0.5% Nonidet P-40). The immunoprecipitate was isolated with protein A- Sepharose beads (Sigma) and washed in NTN before being resuspended in loading buffer for Western blotting. For competition assays, the immunoprecipitate was further incubated in 40 μM GST/GST-HIP₂-72 or PBS control in 2 mM CaCl₂.

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Protein concentrations were determined using the BCA protein assay (Pierce), and equivalent protein concentrations were Western biotied. Western blots were analyzed using densitometry in the imageJ package (www.nih.gov).

Figure 4. Schematic of the Role of Hippocalcin in Synaptically Evoked AMPAR Internalization

Ca²⁺ entry via NMDARs activates the hippocalcin myristyl switch and stimulates binding to β2-adaptin of the AP2 complex, causing translocation of AP2-hippocalcin to the plasma membrane (A). AP2 binds to the GluR2 subunit of AMPARs and recruits clathrin (B). Clathrin displaces hippocalcin prior to endocytosis, after which AMPAR internalization occurs (C).


