

Proteolysis of glutamate receptor-interacting protein by calpain in rat brain: implications for synaptic plasticity

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Abstract

Activation of the calcium-dependent protease calpain has been proposed to be a key step in synaptic plasticity in the hippocampus. However, the exact pathway through which calpain mediates or modulates changes in synaptic function remains to be clarified. Here we report that glutamate receptor-interacting protein (GRIP) is a substrate of calpain, as calpain-mediated GRIP degradation was demonstrated using three different approaches: (i) purified calpain I digestion of synaptic membranes, (ii) calcium treatment of frozen-thawed brain sections, and (iii) NMDA-stimulated organotypic hippocampal slice cultures. More importantly, calpain activation resulted in the disruption of GRIP binding to the

GluR2 subunit of α -amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors. Because GRIP has been proposed to function as an AMPA receptor-targeting and synaptic-stabilizing protein, as well as a synaptic-organizing molecule, calpain-mediated degradation of GRIP and disruption of AMPA receptor anchoring are likely to play important roles in the structural and functional reorganization accompanying synaptic modifications in long-term potentiation and long-term depression.

Keywords: calpain, glutamate, GRIP, receptor, synaptic plasticity.

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Ionotropic glutamate receptors mediate the majority of excitatory synaptic transmission in CNS. Among them, the NMDA and α -amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors play critical roles in the induction and expression of long-term synaptic plasticity in the hippocampus, a proposed cellular basis of learning and memory (Bliss and Collingridge 1993; Malenka and Nicoll 1999). In the past few years, protein phosphorylation has been shown to result in the rapid potentiation of synaptic transmission at excitatory synapses (Barria *et al.* 1997; Yu *et al.* 1997; Carroll *et al.* 1998; Lu *et al.* 1998; Malenka and Nicoll 1999). Moreover, recent studies using green fluorescent protein (GFP)-tagged GluR1 transfection of hippocampal neurons have indicated that long-term potentiation (LTP) involves the rapid insertion of AMPA receptors into post-synaptic membranes, whereas long-term depression (LTD) could be caused by an opposite process (Carroll *et al.* 1999; Shi *et al.* 1999). In particular, reagents that interfere with exocytosis and endocytosis were found to block the formation of LTP and LTD, respectively (Lledo *et al.* 1998; Luscher *et al.* 1999; Man *et al.* 2000). Thus, the hypothesis proposed 15 years ago that LTP was mediated by changes in

the number of synaptic AMPA receptors (Lynch and Baudry 1984) has received strong experimental support.

Although accumulating evidence indicate that AMPA receptors are recycling between a synaptic and an intracellular compartment under both basal conditions and in response to appropriate signals, the detailed mechanisms implicated in synaptic insertion or removal of AMPA receptors remain largely unknown. Functional AMPA receptors do not diffuse freely in post-synaptic membranes but rather are stabilized within a highly dense and specialized structure, called the post-synaptic density

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Abbreviations used: AMPA, α -amino-3-hydroxy-5-methylisoxazole-4-propionate; GFP, green fluorescent protein; GRIP, glutamate receptor-interacting protein; LTD, long-term depression; LTP, long-term potentiation; MEM, minimal essential medium; PSD, post-synaptic density; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

(PSD), by interactions with a family of anchoring proteins (Sheng 1997; Craven and Brecht 1998; Sheng and Pak 1999; Wyszynski *et al.* 1999). Thus, receptor relocalization, at least removal of receptors from PSDs, might require the disruption of their association with anchoring proteins. The glutamate receptor-interacting protein (GRIP) contains several PDZ domains, is enriched in PSDs, and has been suggested to be such an anchoring protein (Dong *et al.* 1997, 1999a,b; Wyszynski *et al.* 1998, 1999; Sheng and Pak 1999). Glutamate receptor-interacting protein was initially identified in the yeast-two-hybrid system by its interaction with the C-terminal domain of GluR2 subunits of AMPA receptors and was later found to interact with AMPA receptors in neurons. It was therefore suggested that GRIP might play a role in AMPA receptor synaptic stabilization. Furthermore, as PSDs contain numerous interconnected proteins, including ion channels, scaffolding proteins, signal transduction-related proteins and cytoskeletal proteins (Ziff 1997; Kennedy 1998; Luscher *et al.* 2000), it is likely that insertion of AMPA receptors into PSDs requires significant structural modifications. Indeed, morphological changes in dendritic spines have been consistently observed in long-term synaptic plasticity (Schuster *et al.* 1990; Geinisman 1993; Trommald *et al.* 1996; Toni *et al.* 1999; Weeks *et al.* 1999; Luscher *et al.* 2000).

Calpains represent a family of calcium-activated neutral proteases and have been shown to be necessary for LTP formation (Oliver *et al.* 1989; Denny *et al.* 1990; del Cerro *et al.* 1994; Vanderklish *et al.* 1996). Calpain has been found in post-synaptic structures (Perlmutter *et al.* 1990) and NMDA receptor stimulation results in calpain activation in dendritic spines (Vanderklish *et al.* 2000). Thus, it is likely that calpain participates in the process of receptor mobilization and synaptic reorganization, possibly by modifying the structure of glutamate receptor anchoring molecules. Here, we report that calpain activation results in the truncation of GRIP, and that such truncation produces the disruption of GRIP/GluR2 subunit interactions.

Materials and methods

Preparation of synaptic membranes and tissue sections

Synaptic membrane fractions were prepared from Sprague–Dawley rat forebrain according to Massicotte *et al.* (1990). Briefly, rat forebrains were homogenized in 0.32 M sucrose containing 10 mM Tris, 1 mM EGTA, 1 mM EDTA, 50 μ M leupeptin, 100 μ M phenylmethanesulfonyl fluoride and 2 μ g/mL aprotinin (pH 7.4), with 10 up and down strokes in a motor-driven glass-Teflon homogenizer to obtain the whole homogenate. Pellets of membrane fractions were obtained by centrifugation of homogenates at 48 000 g for 15 min and resuspension in 0.32 M sucrose. Membranes were washed with ice-cold distilled water containing 2 mM EGTA and 0.1 mM leupeptin and finally resuspended in 100 mM Tris-acetate buffer, pH 7.4, containing 0.1 mM EGTA.

Aliquots were stored at -80°C until the day of use. Alternatively, subcellular fractionation was performed as described by Whittaker *et al.* (1964). In brief, hippocampal homogenates prepared in 0.32 M sucrose buffer containing 10 mM Tris, 0.1 mM EGTA, pH 7.4, were first centrifuged at 1000 g for 10 min to remove the nuclear fraction (P1). The supernatant (S1) was recentrifuged at 10 000 g for 20 min to yield the mitochondrial fraction (P2); S2 was then centrifuged at 100 000 g for 60 min to yield the microsomal fraction (P3) and the cytosolic fraction, S3. P2 and P3 were resuspended in 0.32 M sucrose containing 10 mM Tris, 0.1 mM EGTA, pH 7.4 and they, as well as the S3 fraction were later treated with calpain.

Frozen–thawed tissue sections were prepared following rapid freezing of adult Sprague–Dawley rat forebrains in methyl-butane at -30°C . Coronal 20- μ m thick sections were cut on a cryostat and thaw-mounted onto chrom-alum gelatin-coated slides; they were kept at -70°C until the day of use (generally < 1 week).

Calpain digestion of synaptic membranes and calcium incubation of frozen–thawed brain sections

Synaptic membranes or other subcellular fractions (containing ≈ 300 μ g proteins) were incubated in Tris-acetate buffer with or without 2 mM calcium and calpain I (2.4 units/mL) at 37°C for 30 min. The resulting aliquots were immediately processed for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and western blot. For immunoprecipitation experiments, larger amounts of synaptic membranes (300 μ L, equivalent to 1.5 mg proteins) and a lower calpain concentration (0.6 U/mL) were used. Alternatively, adjacent tissue sections were thawed and incubated in Tris-acetate buffer (100 mM, pH 7.4, containing 0.1 mM EGTA) with or without 2 mM Ca^{2+} at 37°C for 30 min. After incubation, sections were collected and sonicated in 0.32 M sucrose containing 10 mM Tris, 1 mM EGTA, 1 mM EDTA, 50 μ M leupeptin, 100 μ M phenylmethanesulfonyl fluoride and 2 μ g/mL aprotinin (pH 7.4).

Activation of endogenous calpain in organotypical hippocampal cultures

Organotypical cultures of hippocampal slices were prepared using the technique of Stoppini *et al.* (1991) as described previously (Bruce *et al.* 1995; Gellerman *et al.* 1997). In brief, hippocampi were harvested under sterile conditions from 7-day-old Sprague–Dawley rat brains in chilled minimum essential medium (MEM; Gibco no. 61100061) containing 25 mM HEPES, 10 mM Tris-base, 10 mM D-glucose and 3 mM MgCl_2 and placed on a Teflon stage of a McIlwain tissue chopper. Transverse slices (400- μ m thick) were cut and transferred to a humidified membrane insert (Millicell-CM, Millipore, Bedford, MA, USA; 0.4 mm pore size), which was placed in a deep-well plate with 1 mL of MEM (Gibco no. 41200-072) containing 3 mM glutamine, 30 mM HEPES, 5 mM NaHCO_3 , 30 mM D-glucose, 0.5 mM L-ascorbate, 2 mM CaCl_2 , 2.5 mM MgSO_4 , 1 μ g insulin, and 20% horse serum, pH 7.2. In this manner, explants were supported by the membrane at an air–medium interface and kept in an incubator at 35°C with a 5% CO_2 -enriched atmosphere. After 14 days *in vitro*, hippocampal slice cultures were incubated with growth medium without serum, followed by exposure medium (serum-free growth medium containing 20 μ M NMDA and 4 mM CaCl_2) in the presence or

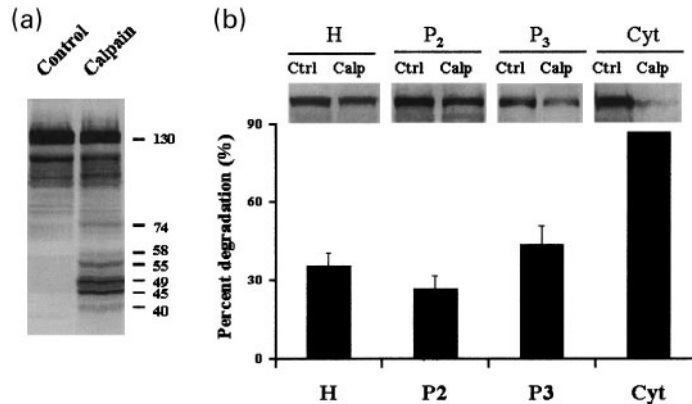


Fig. 1 Calpain-mediated degradation of glutamate receptor-interacting protein (GRIP) in synaptic membranes and different subcellular fractions. (a) Synaptic membranes were incubated with purified calpain I (Calpain, 2.4 U/mL) and calcium (2 mM) at 37°C for 30 min. Membrane proteins were resolved by SDS-PAGE, transferred onto nitrocellulose membranes and probed with anti-GRIP antibodies. Molecular masses of native GRIP, as well as of calpain-generated degradation products, are indicated on the right in kDa. (b) Adult

brains were homogenized (H) and fractionated into P2, P3 and S3 (Cyt) fractions. Aliquots from each fraction were incubated with purified calpain I (Calpain, 2.4 U/mL) and calcium (2 mM) at 37°C for 30 min. (Upper) Western blots showing the degradation of native GRIP species in different fractions (note that different amounts of proteins were loaded for different fractions). (Lower) Quantification of western blots showing the percentage degradation in different fractions. Results are means \pm SEM of three experiments.

absence of 100 μ M calpain inhibitor III for 3 h. The slices were then processed for western blotting.

NMDA treatment of acute hippocampal slices

Transverse hippocampal slices (400- μ m thick) were prepared from 30-day-old Sprague-Dawley rats in oxygenized ice-cold dissection buffer (in mM: sucrose, 212.7; KCl, 2.6; NaH₂PO₄, 1.23; NaHCO₃, 26; glucose, 10; MgCl₂, 3; and CaCl₂, 1, pH, 7.2) using a McIlwain tissue chopper. The slices were transferred gently in a chamber containing artificial CSF (composition in mM: NaCl, 124; KCl, 5; NaH₂PO₄, 1.25; NaHCO₃, 26; glucose, 10; MgCl₂, 1.5; and CaCl₂, 2.5) bubbled with 95% O₂ and 5% CO₂ and were allowed to equilibrate at 30°C for 1 h. Twenty micromolar NMDA was added for 5 min. Slices were collected 15 min after the washout of NMDA and rapidly homogenized in Tris buffer (Tris, 100 μ M; EDTA, 1 mM; aprotinin, 100 μ M; phenylmethanesulfonyl fluoride 100 μ M; leupeptin, 50 μ M; pH, 7.4) and processed for immunoprecipitation.

Immunoprecipitation

Proteins from calpain-treated synaptic membranes (300 μ L, equivalent to 1.5 mg proteins) or from NMDA-treated acute slices (300 μ L, roughly equal to 1.5 mg proteins) were solubilized by adding 2 vol. (600 μ L) of 1.5% deoxycholate solution (1.5% deoxycholate, 50 mM Tris, 2 mM EGTA, 150 μ M leupeptin, 150 μ M phenylmethanesulfonyl fluoride, 3 μ g/mL aprotinin, pH 9.5) followed by incubation at 35°C for 30 min. Following addition of 0.1% Triton X-100 (final concentration), extracts were centrifuged at 24 000 g for 30 min and the supernatant was used for immunoprecipitation. One hundred and fifty microliters of a 1:1 protein A slurry was washed three times in cold Tris buffer (50 mM Tris, 2 mM EGTA, pH 7.4), to which 550 μ L of deoxycholate-solubilized supernatant along with the antibodies of interest were then added (5 μ g GluR2 or 10 μ g of GRIP). The mixture was rotated gently at 4°C overnight. After washing the

protein A beads with 50 mM Tris buffer, pH 7.4 (the first two washes in the presence of 0.1% Triton X-100), 70 μ L sample buffer (see below) was added to the beads and the proteins were eluted by boiling for 5 min.

SDS-PAGE and western blots

Aliquots from different fractions were boiled at 100°C for 5 min in sample buffer (2% SDS, 50 mM Tris-HCl pH 6.8, 10% 2-mercaptoethanol, 10% glycerol and 0.1% bromophenol blue). The method of Laemmli (1970) was used to perform SDS-PAGE. Proteins were then transferred onto nitrocellulose membranes as described by Towbin *et al.* (1979). Nitrocellulose membranes were first blocked with 3% gelatin in Tris-buffered saline (TBS) at room temperature (25°C) for 1 h and incubated overnight at 4°C with primary antibodies against GRIP (1 : 1500 dilution; see Wyszynski *et al.* 1999 for details regarding the generation and characterization of the antibody), NR1 (1 : 200 dilution), α -CaMKII (1 : 5000 dilution), or the N-terminal domains of GluR2 (1 : 1000 dilution) in 1% gelatin prepared in TBS with 0.5% Tween-20 (TTBS). These three antibodies were purchased from Chemicon International Inc. (Temecula, CA, USA). After two washes with TTBS for 5 min, membranes were incubated with alkaline phosphatase-conjugated anti-rabbit (1 : 2000) or anti-mouse (1 : 2000) IgG for 2 h at room temperature; antigens were then visualized with Nitro Blue Tetrazolium and 5-bromo-4-chlor-3-indolylphosphate toluidine salt. Blots were scanned and quantitatively analyzed by densitometry with IMAGEQUANT™ software. Student's *t*-test was used when needed.

Results

Exogenous calpain cleaves GRIP *in vitro*

The initial experiment was carried out by incubating synaptic membranes with purified calpain I (2.4 U/mL) in

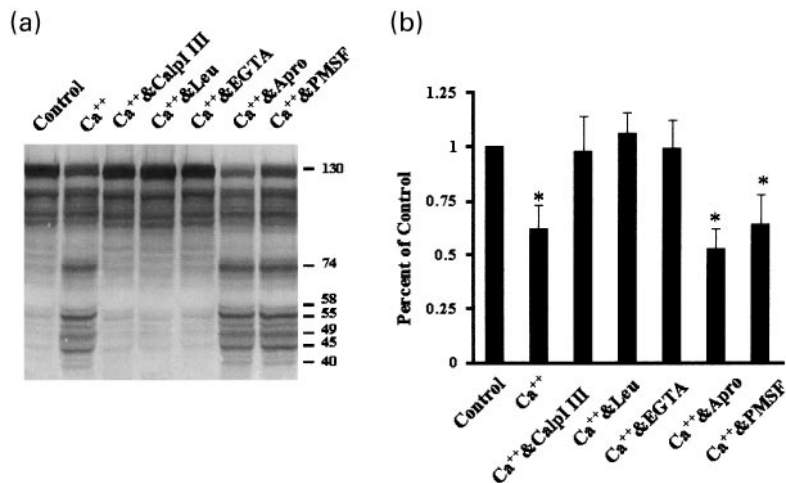


Fig. 2 Effects of calcium treatment of frozen–thawed brain sections on glutamate receptor-interacting protein (GRIP). Frozen–thawed tissue sections (20 μ m) were incubated with 2 mM calcium at room temperature for 30 min, with or without the following protease inhibitors: 100 μ M calpain inhibitor III (Calp I III), 100 μ M leupeptin (Leu), 2 mM EGTA, 50 μ M aprotinin (Apro), 100 μ M phenylmethanesulfonyl fluoride (PMSF). Tissue was collected and processed for western blots using antibodies against GRIP. (a) Representative western blot. (b) Quantification of immunoreactivity of the native species of GRIP (130 kDa) expressed as percentage of control. Results are means \pm SEM of four experiments (* p < 0.05 compared with control, Student's *t*-test).

the presence of 2 mM Ca²⁺ at 37°C for 30 min (Fig. 1). Western blots stained with anti-GRIP antibodies showed a major band migrating with an apparent molecular mass of 130 kDa and a few minor bands with lower molecular masses, in good agreement with previous reports (Dong *et al.* 1997; Wyszynski *et al.* 1998). Following incubation with calpain, the immunoreactivity of the 130-kDa band decreased and several smaller species appeared, demonstrating the existence of multiple truncation sites for calpain. Most prominent were fragments with apparent molecular masses of 74, 55, 49 and 45 kDa. Because the overall immunoreactivity for GRIP remained unchanged between control and calpain-treated samples, it is unlikely that the breakdown products represent unrelated proteins. Because limited proteolysis was observed in similar studies of calpain-mediated degradation of ionotropic glutamate receptors and of the NMDA anchoring protein PSD-95 (Bi *et al.* 1998; Lu *et al.* 2000a), we tested whether partial proteolysis reflected a lower sensitivity of GRIP to calpain. Incubation of synaptic membranes with increasing calpain concentrations showed that GRIP was partially degraded at low calpain concentrations, and that increasing calpain concentration failed to further increase the extent of GRIP degradation (data not shown). To determine whether phosphorylation was involved in protecting GRIP from calpain-mediated truncation, the *in vitro* digestion experiment was repeated in the presence of protein phosphatase inhibitors. Addition of orthovanadate (2 mM), a tyrosine phosphatase inhibitor, or okadaic acid (400 nM), a serine phosphatase inhibitor, however, did not decrease the extent or modify the degradation pattern of GRIP (not shown). We next examined whether different populations of GRIP exhibited different sensitivity to calpain. Adult rat forebrain was homogenized and differential centrifugation was used to obtain various subcellular fractions. When whole homogenate,

P2, P3 and S3 fractions were incubated with purified calpain I, GRIP from the S3 fraction was almost completely degraded, with a truncation of \approx 85% as opposed to 25% for P2 (Fig. 1).

GRIP degradation following activation of endogenous calpain

To study the effect of endogenous calpain activation on GRIP degradation, frozen–thawed rat forebrain sections were incubated with calcium (2 mM) at room temperature for 30 min. The sections were then collected, homogenized and aliquots of homogenates were subjected to SDS–PAGE and western blotting with anti-GRIP antibodies. Calcium incubation resulted in the partial truncation of GRIP in a manner identical to calpain treatment of synaptic membranes, with the appearance of breakdown products with apparent molecular masses of 74, 55, 49 and 45 kDa (Fig. 2). These effects were blocked by EGTA and two calpain inhibitors, leupeptin and calpain inhibitor III. However, cleavage of GRIP following calcium incubation was not blocked by the addition of phenylmethanesulfonyl fluoride or aprotinin (serine protease inhibitors), further confirming that GRIP degradation as a result of calcium incubation was solely caused by endogenous calpain activation.

NMDA treatment of organotypic cultures of hippocampal slices has been shown to activate calpain, and has often been used to investigate mechanisms of synaptic plasticity (Vanderklisch *et al.* 1995; Musleh *et al.* 1997). Several features of the organization and function of the hippocampus are well preserved in such a preparation, thus allowing the effect of calpain activation to be studied under conditions closely matching the *in vivo* environment. Organotypic hippocampal slice cultures were prepared from post-natal day 7 rats; after 14 days *in vitro*, 20 μ M NMDA was added to the medium for 3 h. The cultures were then processed for

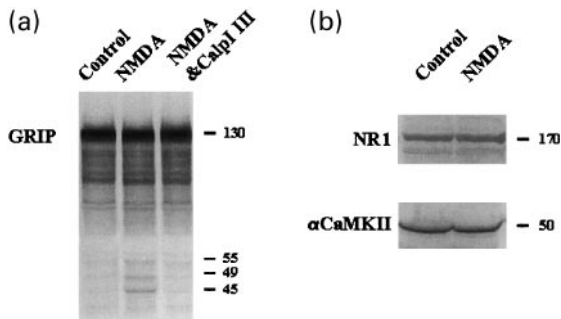


Fig. 3 Effects of NMDA receptor activation in cultured hippocampal slices on glutamate receptor-interacting protein (GRIP). Cultured hippocampal slices were incubated with NMDA (20 μ M), in the presence or absence of calpain inhibitor III (Calp I III), for 3 h. The tissue was collected immediately and processed for western blots using anti-GRIP antibody (a) or anti-NR1, anti- α CaMKII antibodies (b). Results are representative of five experiments.

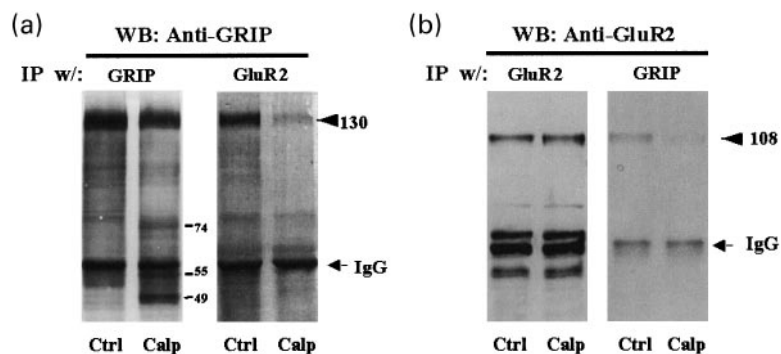
western blots with anti-GRIP antibodies (Fig. 3). Quantification of immunoblots revealed a modest, yet significant ($10.0 \pm 3.4\%$; mean \pm SEM of five experiments; $p < 0.05$, Student's *t*-test compared with control), decrease in immunoreactivity of the native GRIP band and the concurrent appearance of several breakdown products, in particular, the 55-, 49- and 45-kDa fragments. In addition, when the cell permeable calpain inhibitor, calpain inhibitor III (100 μ M), was applied together with NMDA to the culture medium, the decrease in immunoreactivity of the 130-kDa band and the appearance of the breakdown products, were greatly reduced, if not completely blocked. In contrast, several other PSD proteins, including NR1 and α CaMKII, were not degraded under these conditions (Fig. 3).

Calpain-mediated truncation of GRIP disrupts its interaction with AMPA receptors

Previous studies using the yeast two-hybrid system or co-immunoprecipitation of rat brain homogenates demonstrated that GRIP is associated with the GluR2 subunits of AMPA receptors through PDZ domains 4 and 5 both *in vitro* and *in vivo*. Interestingly, most calpain-generated GRIP

degradation products had apparent molecular masses of ≈ 50 kDa. Given that full-length GRIP protein contains 1112 amino acids, it is reasonable to predict that some, if not all, of the calpain truncation sites are located within PDZ domains 4 and 5, suggesting that degradation of GRIP by calpain might disrupt the binding between GRIP and GluR2. In previous studies, we found that GluR2 and GluR3 subunits of AMPA receptors were also truncated following calpain activation (Bi *et al.* 2000; Lu *et al.* 2000b). Because GRIP, but not GluR2 or GluR3, was hydrolyzed at a low calpain concentration of 0.6 U/mL (Fig. 4), we determined the effects of incubating synaptic membranes with this concentration on the interactions between GRIP and AMPA receptors. After incubation with calpain I (0.6 U/mL) at 37°C for 30 min, synaptic membranes were solubilized with 1% deoxycholate and proteins were immunoprecipitated with antibodies against GRIP or GluR2. Following immunoprecipitation with anti-GRIP antibodies, western blots labeled with antibodies against GluR2 and GRIP showed that, under control conditions, a fraction of GRIP was associated with GluR2 (Fig. 4). A similar result was obtained following immunoprecipitation with anti-GluR2 antibodies, in good agreement with previous reports (Dong *et al.* 1999a, b; Wyszynski *et al.* 1999). Following incubation with calpain, GRIP was partially hydrolyzed and GRIP antibodies successfully precipitated both the 130 kDa native species as well as several breakdown products (Fig. 4). As expected, GluR2 was not degraded significantly under such conditions, as GluR2 antibodies precipitated the same amount of GluR2 before and after calpain incubation. However, the interaction between GRIP and GluR2 was reduced dramatically, as very little GRIP co-immunoprecipitated with GluR2 and none of the GRIP breakdown products were observed to be associated with GluR2. Furthermore, whereas the levels of native GRIP were decreased by $35 \pm 6\%$ (mean \pm SEM of three experiments) following incubation with calpain, the levels of GluR2 co-immunoprecipitated with GRIP were reduced by $67 \pm 23\%$ (mean \pm SEM of three experiments).

Fig. 4 Disruption of GluR2–glutamate receptor-interacting protein (GRIP) interaction following calpain-treatment of synaptic membranes. Following incubation with calpain I (0.6 U/mL) at 37°C for 30 min, synaptic membranes were solubilized in 1% deoxycholate and immunoprecipitation (IP) was performed using anti-GRIP or anti-GluR2 antibodies. The precipitated immunocomplexes were resolved by SDS–PAGE and probed with anti-GRIP (a) or anti-GluR2 (b) antibodies. Results are representative of three experiments.



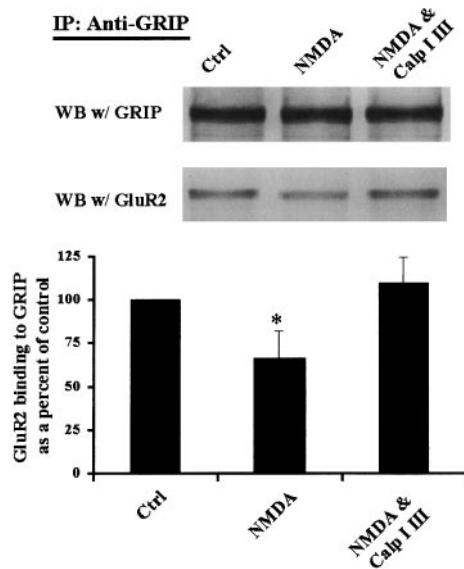


Fig. 5 Effects of NMDA treatment of acute hippocampal slices on GluR2–glutamate receptor-interacting protein (GRIP) interaction. Following incubation of acute hippocampal slices with NMDA (50 μM) at 30°C for 5 min, slice homogenates were solubilized in 1% deoxycholate and immunoprecipitation (IP) was performed using anti-GRIP antibodies. The precipitated immunocomplexes were resolved by SDS–PAGE and probed with anti-GRIP or anti-GluR2 antibodies. (Upper) Representative western blots. (Lower) Quantification of the blots. Results are means \pm SEM of three experiments (* $p < 0.05$ compared with control, Student's *t*-test).

Acute hippocampal slices were incubated with 50 μM NMDA for 5 min, a manipulation that has been reported to produce a long-lasting increase in synaptic efficacy in CA1 as well as calpain activation (Broutman and Baudry 2001). Co-immunoprecipitation with anti-GRIP or anti-GluR2 antibodies was performed to determine the extent of GRIP/GluR2 interaction. No significant differences in the amount of precipitated native GRIP (Fig. 5) or GluR2 (not shown) proteins between control and NMDA-stimulated conditions were observed, suggesting that GRIP or GluR2 degradation, if it occurs, is very limited following a brief NMDA receptor stimulation. In contrast, the interaction between GRIP and GluR2 was decreased by $\approx 40\%$ (Fig. 5). This effect was completely blocked by calpain inhibitor III.

Discussion

Our results indicate that GRIP is a substrate for calpain and that calpain-mediated cleavage of GRIP is likely to occur *in vivo* in response to an increase in intracellular calcium concentration and NMDA receptor activation. We used three strategies to study the effects of calpain activation on GRIP properties. First, GRIP was degraded significantly by purified calpain I in rat forebrain synaptic membranes.

Second, the same degradation pattern was observed following incubation of frozen–thawed brain sections with calcium, indicating that activation of endogenous calpain produces the truncation of GRIP. Finally, and more importantly, NMDA treatment of organotypic hippocampal cultures also led to calpain-mediated degradation of GRIP and the formation of truncation products with the same molecular masses as those observed in the calpain treatment experiment. In every case, the involvement of calpain was assessed by the addition of specific calpain inhibitors and of noncalpain protease inhibitors. In every case, GRIP degradation was only partial and limited, although it was much more extensive in soluble than membrane fractions. The difference between the different calpain sensitivity of these two populations of GRIP is not clear. A possible explanation could be that the presence of multiple interactions between GRIP and other proteins in the PSD limits the access of calpain to GRIP. Alternatively, differences in post-translational states could account for the difference.

As reported previously (Wyszynski *et al.* 1998; Dong *et al.* 1999a, b), GRIP is enriched in synaptic membranes of rat hippocampus and coprecipitates with GluR2 subunits of AMPA receptors. The GRIP–GluR2 interaction was markedly disrupted by calpain activation in synaptic membranes. Interestingly, although only a relatively small fraction of GRIP (35%) appeared to be truncated by calpain, almost all the interaction ($> 70\%$) between GRIP and GluR2 was disrupted. Furthermore, we also demonstrated that a brief NMDA application (5 min) in acute hippocampal slices produced calpain activation and disruption of the GRIP–GluR2 interaction. Several possibilities could account for the lack of GRIP degradation under these conditions. First, it is possible that only a small fraction of GRIP was degraded, an effect too small to be detected by our assay. Indeed, even following prolonged NMDA application in organotypic hippocampal cultures, GRIP degradation was also very limited. Given that the same discrepancy between the level of GRIP degradation and percentage decrease in GRIP–GluR2 binding also occurred in calpain-treated synaptic membranes, these data suggest that calpain might preferably truncate the fraction of GRIP associated with GluR2 subunits of AMPA receptors. Alternatively, other proteins could participate in the interactions between GRIP and GluR2 and these additional proteins could themselves be calpain substrates. It might therefore be interesting to test whether the recently described GRASP (Ye *et al.* 2000) is also a calpain substrate. In addition, adaptin has been reported to be associated with GluR2 subunits of AMPA receptors (Man *et al.* 2000) and was previously found to be a substrate for calpain (Sato *et al.* 1995).

The induction of two forms of long-term synaptic plasticity, LTD and LTP, requires NMDA receptor activation, followed by transient elevations of post-synaptic

calcium concentration (Bliss and Collingridge 1993; Malenka and Nicoll 1999). Downstream effects of elevated calcium include the activation of the calcium-dependent protease calpain as well as of phosphatases (LTD) or kinases (LTP) (del Cerro *et al.* 1994; Barria *et al.* 1997; Musleh *et al.* 1997; Malenka and Nicoll 1999; Lee *et al.* 2000). Several studies have also shown that calpain activation was necessary for LTP formation (Staubli *et al.* 1988; Oliver *et al.* 1989; Denny *et al.* 1990; del Cerro *et al.* 1994; Vanderklish *et al.* 1996). However, the precise pathway by which calpain participates in the expression of long-term synaptic plasticity remains unclear. We previously reported that GluR1 and GluR2 subunits of AMPA receptors were truncated by calpain activation, and that the truncated species of GluR1 and GluR2 were removed from PSDs, possibly for internalization and further degradation (Lu *et al.* 2000b). We further postulated that this would bias synaptic strength toward LTD. Our results show that the AMPA receptor anchoring protein GRIP is also a substrate for calpain and that calpain-mediated truncation of GRIP, GluR2 or some other associated proteins, disrupts GRIP–GluR2 interactions. On the one hand, this could facilitate the removal of the receptors from the synapses and interrupt the insertion of new receptors into synaptic membranes, because one possible function of GRIP is to sort and transport AMPA receptors to synaptic sites (Dong *et al.* 1999a, b). Such an effect could possibly contribute to LTD expression. Indeed, it has recently been shown that GluR2–GRIP interaction is required to activate silent synapses in the spinal cord, probably by recruiting more AMPA receptors to the synapses, and disruption of GluR2–GRIP interaction was also found to be associated with LTD in cerebellum (Li *et al.* 1999; Matsuda *et al.* 2000). On the other hand, given that morphological changes, such as perforated synapses, synaptic splitting and budding (Trommald *et al.* 1996; Toni *et al.* 1999; Luscher *et al.* 2000) have consistently been observed in the expression or consolidation phase of LTP and because GRIP was found to form polymeric complexes as well as to bind to several PSD proteins (Dong *et al.* 1997; Torres *et al.* 1998; Ye *et al.* 2000), it is likely that GRIP could function as a synaptic organizing protein. In this way, degradation of GRIP by calpain could possibly be involved in structural modifications observed in LTP. In addition, truncation of GRIP, as well as of two other PSD proteins, PSD-95 and spectrin, by calpain (Lynch and Baudry 1987; del Cerro *et al.* 1994; Lu *et al.* 2000a), could potentially facilitate the insertion of receptors by opening the PSD and making the post-synaptic membranes more accessible to secretory vesicles carrying AMPA receptors. In this regard, the effect would be permissive and some other triggering signals might be required to initiate the exocytotic machinery.

In conclusion, our results indicate that GRIP is a substrate of calpain and that calpain activation, by degrading GRIP or

some other GRIP-associated protein, eliminates GRIP–GluR2 interaction. This effect is likely to play an important role in the structural and functional reorganization accompanying synaptic modifications observed in LTP and LTD.

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